ASSESSMENT OF THE IMMUNOGENICITY OF PORCINE CIRCOVIRUS 2 (PCV2) VACCINES: A PROTOTYPE VACCINE AND A LAMBDA DISPLAY VACCINE

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ABSTRACT

Porcine *Circovirus* 2 (PCV2) associated diseases (PCVAD) cause economic loss to the global swine industry. Control measures for PCVAD largely depend on the use of PCV2 vaccines. The available commercial PCV2 vaccines contain either inactivated whole virus particles or recombinant PCV2 capsid protein. These preparations most likely contain varying amounts of immune-irrelevant proteins that can cause adverse injection site reactions, with compromised efficacy due to alteration of protective immune epitopes arising during the viral inactivation process. Other constraints include high production cost attributed to propagation of slow growing virus and expression and extraction of recombinant proteins, a requirement for adjuvants, and the induction of a Th2-biased immune response. Hence, development of new PCV2 vaccines is necessary.

There are two recommended PCV2 vaccination strategies. They are i. vaccinating sows, which relies on the passive transfer of maternal immunity to offspring, and ii. immunizing young piglets to induce an active immune response. The piglet vaccination has been shown to confer better protection from mortality. Maternal antibody interference to the induction of an active immune response is an obstacle when piglets are vaccinated at an early age. Can we sidestep this maternal antibody interference? To address this issue, I investigated whether a prototypical PCV2 vaccine, parenterally administered, could override maternally-derived PCV2 antibodies in seropositive piglets. The results of this study were not conclusive. However, they laid the foundation for future studies based upon using varying levels of vaccine antigen with different adjuvants, and administered to piglets with defined maternally derived PCV2 antibodies.

Subsequently, I examined if a new PCV2 vaccine candidate comprised of bacteriophage lambda particles displaying part of the PCV2 capsid protein could induce anti-PCV2 immunity. Initial experiments showed that pigs do not have pre-existing anti-lambda antibodies and thus will not neutralize display particles used as a vaccine at primary vaccination. I produced and characterized lambda phage particles displaying four immunodominant regions of porcine *circovirus* 2 (PCV2) capsid protein fused to the lambda capsid protein D i.e., D-CAP, phage display particles. Expression of D-CAP in *Escherichia coli* (*E. coli*) and its presence in the vaccine preparation was shown by ELISA and Western blots using anti-PCV2 polyclonal antiserum from a gnotobiotic pig. The vaccine, lambda particles displaying PCV2 capsid protein

immunogenic epitopes fused to lambda D protein (LDP-D-CAP), administered without an adjuvant induced both humoral and cellular immunity to PCV2 in conventional pigs, as shown by ELISA, Western blots, virus neutralization assay and delayed type hypersensitivity (DTH) reactions. This work produced the first potential phage vaccine to PCV2. In order to further investigate the feasibility of using the lambda display technology. I produced and characterized two additional lambda display particle preparations, LDP-D-FLAG and LDP-D-GFP, displaying a FLAG tag and the green fluorescent proteins, respectively.

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LIST OF ABBREVIATIONS

aa	Amino acid
b.wt.	Body weight
CMI	Cell-mediated immunity
D-CAP	Immunodominant regions of porcine *circovirus* 2 capsid protein fused to the lambda head protein D
D-FLAG	FLAG tag protein fused to lambda head protein D
D-GFP	Green fluorescent protein fused to lambda head protein D
DTH	Delayed type hypersensitivity reactions
ELISA	Enzyme-linked immunosorbent assay
cELISA	Competitive ELISA
iELISA	Indirect ELISA
LDP	Lambda display particles
LDP-D-CAP	Lambda particles displaying D-CAP
LDP-D-FLAG	Lambda particles displaying D-FLAG
LDP-D-GFP	Lambda particles displaying D-GFP
H & E	Haematoxylin and Eosin stain
hpi.	Hour/s post infection
IHC	Immunohistochemistry
IPMA	Immunoperoxidase monolayer assay
IFA	Immunofluorescence assay

MW	Molecular weight
MDA	Maternally-derived antibodies
nt.	Nucleotide
OD	Optical density
ORF	Open reading frame
Ori	Origin of replication
PCV	Porcine circoviruses
PCV1	Porcine *Circovirus* 1
PCV2	Porcine *Circovirus* 2
PCVAD	Porcine *Circovirus* 2 associated diseases
PMWS	Post-weaning multisystemic wasting syndrome
pfu	Plaque forming unit
PBS	Phosphate buffer saline
PDB	Phage dialysis buffer
PK-15	Pig kidney 15 cell line
TTV	Torque teno virus
UTR	Untranslated region
$TCID_{50}$	50% Tissue Culture Infective Dose
VNA	Virus neutralization assay
VLP	Virus-like particles
WB	Western blot

CHAPTER 1. INTRODUCTION

1.1. CIRCOVIRIDAE

The family *Circoviridae* is classified into three genera of plant and animal viruses, *Circovirus*, *Gyrovirus* and *Anellovirus* [1]. Circoviruses are non-enveloped icosahedral virus particles with single-stranded circular DNA genome [2]. Members of the Circovirus genus include three plant viruses i.e., Banana bunchy top virus (BBTV), Coconut foliar decay virus (CFDV) and Subterranean clover stunt virus (SCSV), two porcine viruses namely porcine *Circovirus* type 1 (PCV1) and type 2 (PCV2) and several avian viruses such as Psittacine beak and feather disease virus (BFDV), Columbid or Pigeon circovirus (CoCV or PiCV), Canary circovirus, Goose circovirus (GCV) and duck circovirus (DuCV) [1, 3-6]. Chicken anaemia virus (CAV) is the only member of the *Gyrovirus* genus. Torque teno virus (TTV) and Torque teno mini virus (TTMV) are classified into the *Anellovirus* genus [1].

1.2. Torque teno virus (TTV)

Torque teno virus (TTV) is circular single stranded non-enveloped DNA virus (ssDNA), similar in structure to porcine Circoviruses, identified in humans, pigs, poultry, cattle, sheep, dogs and cats [1, 7]. Three open reading frames (ORFs) were described for TTV genome characterized for pigs i.e., ORF1=635 amino acids (aa) capsid protein, ORF2=73 aa replication protein, ORF3=224 aa (of unknown function) [7, 8]. The size of a cloned pig TTV genome, named Sd-TTV31 (GenBank accession # AB076001.1), is 2878 nucleotides (nt.) which included an untranslated region (UTR) of ~24% nt. containing regulatory sequences required for transcription and replication [7, 8]. Phylogenetic analysis of TTV genome from pigs and other domestic animal species revealed species specificity. Two genotypes of pig TTVs were identified with nucleotide (nt.) sequence homology varying between 44-70%. However, greater homology was observed among pig isolates from the same geographic region [7]. No evidence of a TTV link to human disease has been found [7]. Inoculation of gnotobiotic pigs first with TTV and subsequently by PCV2 was shown to induce post-weaning multisystemic wasting syndrome (PMWS) [9] indicating a likely role of TTV in pathogenesis of PMWS. A higher prevalence of TTV infection was reported in PMWS affected pigs than healthy pigs [10].

1.3. PORCINE CIRCOVIRUSES (PCVs)

1.3.1. PCV genome organization

PCVs are 16-18 nm in diameter containing a genome of approximately 1760 nt. They are the smallest mammalian viruses yet known and encode two major open reading frames, rep and cap [3, 5, 11]. The respective gene products, Rep and Cap perform most elementary viral functions namely, replication and packaging of the viral genome. The rep and cap genes are oriented head-to-head and the resulting ambisense genome organization creates two intergenic regions (IR), a shorter region between 3′-ends of *rep* and *cap* and a larger one between their 5′ ends [3, 11]. The latter IR is comprised of origin of replication (Ori) characterized by stem loop structure with a nonamer 5′ T/AAGTATTAC sequence at its apex flanked by an inverted repeat (palindrome) of 11 nt. and three adjacent hexamer 5'-CGGCAG motifs [3]. The 3′-part of the stem and the two proximal hexmers serve as the binding sites for replicases. The stem loop structure is indicated as a common feature of viruses that replicate via rolling-circle replication (RCR) [3, 11]. The nonamer sequence 5′ T/AAxTAxTAC at the apex of the loop is termed the essential core element (ECE) as it plays a key role in PCV replication and stable infectious virus production while "x" positions can be substituted without functional loss [12].

1.3.2. PCV proteins/replication

The largest ORF, ORF1 or *rep* is on the viral plus-strand transcribed clockwise into two collinear replicase proteins i.e., Rep, full length transcript (PCV1: 312 aa; PCV2: 314 aa) and Rep′, a truncated and C-terminal frame-shifted transcript (PCV1: 168 aa; PCV2: 178 aa). Both Rep and Rep′ are necessary to initiate PCV replication [3, 5, 11]. Rep recognizes the right arm of the palindrome while Rep′ binds to the two proximal tandem repeat hexamers (H1 and H2) at the Ori [5]. Moreover, Rep and Rep′ of PCV1 and PCV2 can recognize their respective binding sites interchangeably between these two viruses and initiate DNA replication [13]. Promoter of *rep* (Prep) is negatively regulated by Rep on binding to two inner hexamers (H1 and H2). However, Rep′ does not exert such repression of Prep attributed to differential capability of Rep and Rep′ to interact with transcription factors [5].

ORF2 or *cap* is on the minus-strand transcribed counter clockwise into the major structural capsid protein, Cap (PCV1: 232 aa; PCV2: 233 aa) promoter (Pcap) which is mapped

within the *rep* gene [3, 11]. Rep, Rep′ or Cap do not influence the transcription initiated at Pcap. Cap is the main antigenic determinant of PCV [14, 15] and, has a molecular weight (MW) ~28 kDa [3, 16]. It contains an arginine-rich basic N-terminus which is highly conserved among PCV2 isolates and is believed to have DNA binding activity in the native virion [3, 17, 18]. Synthesis of PCV2-Cap in an insect cell line (Sf9) using a baculovirus expression system demonstrated formation of empty capsid heads referred to as "virus like particles" [19]. There are two additional ORFs (ORF3 and ORF4) described on the PCV genome both of which mapped within ORF1 and are transcribed counter clock wise (opposite of ORF1 transcription direction) [11, 17]. Expression of ORF3 was shown in PCV2 infected PK-15 cells and found to play a role in virus induced cell death by apoptosis [20]. Transfection experiments with an ORF3 deficient PCV2 mutant showed that the protein is not essential for viral replication.

Rep, Rep′ and Cap carry nuclear localization signals (NLS). In PCV infected cells, Rep and Rep′ co-localized in the nucleoplasm. In contrast, Cap is localized in the nucleoli in the early stage of the infection and later migrates to the nucleoplasm and is then exported into the cytoplasm. The intracytoplasmic inclusions were found dispersed throughout the cytoplasm but were most numerous in the perinuclear cytoplasm [21]. Since all PCV-encoded proteins localize in the nucleus, encapsidation of the viral genome is considered to occur in the nucleus [11].

1.3.3. Genetic differences between PCV1 and PCV2

Analysis of genetic variations between PCV1 and PCV2 revealed a greater nt. homology, ~80%, between *rep* genes compared to *cap* with ~62% homology [3, 16]. Overall nt. homology was 68% between PCV1 and PCV2 isolates [22] forming two distinct clusters by phylogenetic analysis [23]. These analyses showed that PCV1 and PCV2 are genetically different. Overall nt. sequence comparison among PCV2 isolates revealed > 94% homology [17, 24]. The nt. homology was much greater among PCV2 isolates within a geographic region; homology among European and North American isolates were 99.8% and 99.5% respectively [17]. Amino acid sequence comparison among PCV2 isolates between Rep and Cap proteins was 99-100% and 90-100% respectively [25, 26]. Amino acid sequences analysis of the Cap protein of PCV2 isolates from healthy and diseased pigs recognized three regions of heterogeneity i.e., 59-80, 121-136 and 180-191 [25] and the first two regions correspond with two of the immunodominant regions identified by Pepsacan analysis [14]. It was speculated that these regions might be exposed to

selective immune pressure and, therefore, are candidate regions for involvement in emergence of new PCV2 variants. However, these studies were unable to identify a molecular marker for PCV2 pathogenicity, as similar aa sequence patterns were found in isolates from both healthy and sick pigs. Further, origin of replication and rep genes of PCV1 and PCV2 are highly conserved, replication proteins are functionally exchangeable and replication efficiency is comparable *in vitro*. Therefore, the replication strategy of the two viruses is not the key element determining their distinct pathogenicity [13]. These data indicate that the PCV2 genome, notably rep gene, is relatively stable.

A unique restriction enzyme site *Nco*1 is present only in the PCV2 genome. A universal PCR-restriction fragment length polymorphism assay (PCR-RFLP) was developed based on this feature to distinguish PCV1 and PCV2 genomes. The assay yields two fragments for PCV2 compared to single undigested PCV1 genome following digestion with *Nco*1 enzyme. Detection of three fragments is indicative of the presence of both PCV1 and PCV2 DNA in the sample [18].

1.3.4. Pathogenicity differences between PCV1 and PCV2

PCV1 was first discovered as a contaminant in the pig kidney PK-15 cell line (ATCC-CCL31) [27], and was later shown to be non-pathogenic in rabbits, mice and pigs [28-30] and is not linked to any disease condition. In contrast, PCV2 was isolated initially [31] and subsequently world-wide, from diseased pigs suffering from what was later described as post-weaning multisystemic wasting syndrome (PMWS) [26, 32-36]. Experimental inoculation of pigs with PCV2 confirmed its pathogenicity [28, 37-46]. The difference in pathogenicity between PCV1 and PCV2 was not linked to any specific genetic differences between the two viruses [47].

1.3.5. Genetic differences among PCV2 isolates and their emergence

PCV2 DNA was detected in both PMWS-affected (100%) and healthy pigs (76%) [24]. Further, multiple PCV2 strains were detected in a single pig, most often in PMWS affected pigs [48, 49]. Studies demonstrated evidence of emerging new PCV2 variants as a result of recombination events with simultaneous multiplication of multiple PCV2 strains in a single host animal [48-50] or a cell line [51]. Propagation of viral inoculums prepared from PMWS-affected

pigs in PK-15 cells showed the emergence of a perfect mosaic PCV2 strain as a result of recombination events that occurred between two parental strains in the *rep* and *cap* genes [51]. Nucleotide sequences of three PCV2 strains isolated from PMWS-affected pigs from different farms were compared to 164 PCV2 nucleotide sequences obtained from GenBank to determine their phylogenetic origin [50]. Evidence for potential recombination events was analysed using recombination detection software (RDS) package [50]. These analyses revealed that the three test strains fell into two of the seven parental PCV2 lineages, named A through G, providing evidence for natural recombination events occurred among different PCV2 strains. The potential recombination sites were identified within the origin of replication and the *rep* gene. Similar phylogenetic analysis with nt. sequences of 148 PCV2 isolates demonstrated a high degree of homology, yet formed into two major groups, groups 1 and 2 consist of eight sub-clusters i.e., 1A-1C and 2A-2E [23]. The RDS programming revealed that the PCV2 strains within cluster 1B evolved by recombination events between isolates from clusters 1A and 2D. The two PCV2 groups differ from each other in their total number of nt. sequences; PCV2-group 2 have 1768 nt. compared to PCV2-group 1 with a single nt. deleted at 1040 position in the C-terminal of cap gene resulting in a change in aa sequence at 232 position, i.e., K232N [24, 52]. Further, each group has stretches of distinct nt. and aa sequences, and signature motifs [52]. Analysis of nucleotide sequence for the *cap* gene of PCV2 strains from PMWS affected and non affected herds revealed consistent detection of PCV2-group 1 in the former group, while the latter reported exclusively genogroup 2 [48]. PCV2-group 1 was detected in the United States for the first time during a severe outbreak of PMWS during 2005-2006 [52]. Simultaneously, a dramatic increase in PCV2-group 1 associated deaths was reported in Canada [53]. These observations led to speculation that the more pathogenic PCV2 strains belong to PCV2-group 1. In contrast, other studies could neither identify a direct link between a particular PCV2 genogroup and the development of PMWS [48, 54], nor any PCV2 molecular marker linked to virulence and, therefore, associated with severe PMWS outbreaks [24, 25]. However, a PCV2 strain with point mutations on the capsid protein, raised following serial passage in PK-15 cell culture, had significant attenuation of virulence following inoculation into 3-4 weeks old specific-pathogen free (SPF) pigs. This finding is indicative of the presence of likely virulent determinants on the PCV2 capsid protein (PCV2-Cap) [55]. Germ-free pigs inoculated with varying doses of either PCV2 group 1 or -2 had similar pathology and mortality rates (~ 50% mortality) [56].

Gnotobiotic pigs administered PCV2-group 1 and -2 alone, or in combination with keyhole limpet hemocyamin in incomplete Freund's adjuvant (KLH/ICFA), did not develop clinical disease and lesions were indistinguishable among the groups [57]. However, overt PMWS was produced when gnotobiotic pigs were inoculated with either group of virus initially by intraperitonial route, followed by intransal administration of a heterogonous strain 1 week later, irrespective of the order of administering the inoculums [58]. Gross and histological lesions were significantly greater in the heterologous challenge groups compared to groups that received single or double homologous inoculums. These findings suggest that the recent upsurge of PMWS cases in North America could be due to dual heterologous infection [58]. Nomenclature was established to designate different PCV2 groups. PCV2-group 1 that were to have caused severe PMWS outbreaks in recent years was classified as PCV2b and PCV2-group 2 that was discovered in 1997 and continued to be encountered in both clinical and healthy pigs was classified as PCV2a [48, 59]. A vaccine containing PCV2-Cap of a genotype 2 had shown cross protection against heterologous strains originated from two geographic regions i.e., Europe and North America [60], indicating genetic differences among PCV2 isolates do not likely affect critical protective epitopes.

1.3.6. PCV life cycle

PCV2 antigens are predominantly detected in monocyte/macrophage lineage cells and follicular dendritic cells in infected pigs [61-63]. *In vitro*, both PCV1 and PCV2 are most commonly propagated in porcine kidney (PK-15) cells [27, 64-66]. Therefore, the mechanism of PCV2 attachment and internalization kinetics have been studied by *in vitro* infection experiments using a porcine monocytic cell line, 3D4/31 and PK-15 cells, and using PCV2 or PCV2 virus like particles (VLP) [67-69]. Glycosaminoglycans, such as heparin sulfate (HS) and chondroitin sulfate B (CS-B) were shown to serve as the cell surface receptors on 3D4/31 and PK-15 cells [67]. This study constituted several experiments to demonstrate specific binding of PCV2 to immobilized heparin (heparin-Sepharose column), reduction of binding on the cell surface and percentage of infected cells by prior incubation of target cells with heparin, HS and CS-B due to competition or enzymatic treatment of cells to remove cell surface HS and CS-B. The findings were confirmed by demonstrating marked reduction of PCV2 infection in a mutant cell line deficient in HS and CS-B compared to wild type PCV2 competent Chinese hamster ovary (CHO)

cells. The finding that HS and CS-B serving as cell surface receptors is more relevant since PCV2-Cap is reported to contain a putative heparin binding motif [67]. However, finding that HS and CS-B deficient cells were unable to abrogate PCV2 infection completely led to investigation of other mechanisms of viral entry into cells, such as clathrin- or caveolae-mediated endocytosis and macropinocytosis. In one such study in 3D4/31 cells, there was co-localization of PCV2 VLP and clathrin by double immunofluorescence staining [69]. Further, this study demonstrated a significant decline in PCV2 infection by chemical inhibition of clathrin-mediated endocytosis, cellular actin polymerization and endosomal acidification using lysomotrophic weak bases such as ammonium chloride and chloroquine diphosphate (CQ), but not by inhibition of caveolae-mediated endocytosis or macropinocytosis. Cellular actin polymerization plays a positive role in clathrin-mediated endocytosis [70] and gradual pH drop in the endosomal maturation process, i.e., early endosomal pH of 6.0-6.8 drops to ~5.0 in lysosomes, is reported to enhance uncoating and escape of the virus into cytoplasm [69]. The effect of the inhibition of endosome-lysosome system on PCV2 infection was further investigated using several epithelial cell lines [68]. In contrast to the findings with the porcine monocytic cell line 3D4/31, PK-15 cells showed an increased PCV2 infection following the inhibition of endosome-lysosome pH during the early stage of infection (up to 6 hr) [68]. This effect was not observed when cells were treated with CQ 1 hr prior to or 12 hr after the infection. Further investigation revealed that serine proteases increase PCV2 infection in both 3D4/31and PK-15, irrespective of pH alterations in the endosome-lysomes compartment, by aiding the disassembly of viral capsid. In summary, heparin sulfate and chondroitin sulfate B serve as the cell surface receptors for PCV2 attachment. Virus is internalized by clathrin-mediated endocytosis dependent on the cellular actin polymerization process. Serine protease dissembles PCV2-Cap and aids in uncoating the virus. Single stranded (ss) DNA is transported into the nucleus and converted by host DNA polymerases into the double stranded (ds) intermediate. The rep and cap mRNAs are transcribed, proteins are synthesized and imported into the nucleus. Rep and Rep′ bind to dsDNA to initiate DNA replication by rolling circle replication (RCR) [4]. Assembly and release of PCV virions are not yet studied [11].

1.3.7. Biological and Physico-chemical properties of PCV

The PCV2 particle, has a diameter of ~20.5 nm and an icosahedral structure with 60 capsid protein molecules arranged in 12 pentamer units [71]. PCV1does not haemagglutinate erythrocytes from a wide range of species, has buoyant density of ~1.37 g/ml in a CsCl gradient, is resistant to treatment with chloroform, is stable at pH 3.0, and during incubation at 56^{o}C or 70^{o}C for 15 min [6, 72]. PCV can be disrupted by heating at 100^{o}C for 1 min with Sodium dodecyl sulphate (SDS) and mercaptoethanol to demonstrate proteins by polyacrylamide gel electrophoresis [27].

1.3.8. Propagation, isolation and purification of PCV

Several cell lines originating from different animal species were shown to be permissive to PCV infection [21, 67-69, 72, 73]. However, only pig-derived and Vero cell cultures showed an increase in viral titers following the second passage of the virus. Vero cells were derived from fibroblasts from an African Green monkey. The other cell lines examined showed a reduction or elimination of viral infection following the second passage of the virus [72]. The porcine kidney (PK-15) cell line is the most commonly used cell culture in propagation of PCV [27, 64-66]. A study on the kinetics of PCV2 replication in PK-15 cells revealed an increase in viral antigens, RNA transcripts and progeny viruses in a time dependent manner. Considerable differences in replication kinetics were observed among PCV2 strains tested. Using polyclonal antiserum, viral antigens of Stoon-1010 isolate of PCV2 were detected in the cytoplasm starting from 12 hr post-infection (hpi), and reaching a maximum percentage of virus positive cells (1.8%) by 48 hpi. A rise in the viral titer in the culture supernatant was observed at 36 hpi and coincided with appearance of the first foci of infected cells, indicating a duration of 24-36 h for the full replication cycle of PCV2 in PK-15 cells [74]. Both Rep and Cap proteins were detected in the nucleus at 12 hpi by using double immunofluorescence staining. PCV2 does not result in cytopathic effects [75]. However, the numbers of detaching cells that contain Cap abundantly present in their cytoplasm increased starting from 48 hpi [74]. The number of PCV2 infected cells was increased by 50 fold following treatment of the cell culture with d-glucosamine-HCl at 4-6 hpi. PCV DNA replication depends on cellular enzymes expressed during the S phase of growth, and DNA replication starts only once the cells have passed mitosis. It was shown that PCV DNA, by itself, is incapable of penetrating the nuclear membrane and, therefore, must be

included in the daughter nuclei following mitosis. Glucosamine is reported to aid the entry of PCV DNA in to the cell nucleus [76]. However, PCV2 virus titers do not generally exceed 10^5 $TCID_{50}$ per ml when the virus is propagated in PK-15 cells [61, 65, 66]. Hence, a number of studies have used PCV2 virus-like particles in place of intact virus for experiments that required large amounts of viral protein [67-69]. The low viral titers in PK-15 cells were reported to be because of its heterogeneous cell population, i.e., constituted of cells with varying permissiveness to PCV2 [65]. Therefore, a new cell line (PK-C1) comprised of a homogenous high permissive cell population was generated following a selection procedure for permissive cells from parental PK-15 cell culture. The new cell line was reported to produce higher viral yield (10^8 $TCID_{50}$ per ml) [65]. PCV1 and PCV2 DNA were also shown to be infectious and produce progeny virus in PK-15 cell cultures [17, 27, 76, 77]. Cell culture grown virus can be isolated following three freeze/thaw cycles of infected cells and initial low speed centrifugation followed by ultracentrifugation of the supernatant. The pellet is suspended in phosphate buffer saline (PBS, pH 7.2) and a follow up sucrose or CsCl gradient centrifugation used to purify the virus [27, 65, 72, 78].

1.3.9. Detection & quantification of PCV antigens/proteins

PCV antigens on acetone-fixed tissue impressions, cryostat sections, formalin-fixed paraffin-embedded tissues or viral infected tissue cultures can be detected by immunohistochemistry using PCV1 or 2 specific mAbs [31, 39, 79, 80]. PCV DNA in tissue sections can similarly be detected by *in situ* hybridization using virus specific probes [31, 80-82]. Indirect immunofluorescence [79], immunoperoxidase monolayer assay (IPMA) [83] and antigen capture ELISA [79, 84] methods are employed to detect and quantify viral antigens. Polymerase chain reaction (PCR) methods are widely used to detect PCV DNA in formalin-fixed tissues [85], semen [86] blood/serum/plasma [87-90], faeces and other excreta [91, 92]. PCV proteins are demonstrated by heating purified virus in a reducing buffer at 100^{o}C for 1 min [27] or 95^{o}C for 5 min [93] to disrupt the particles, followed by SDS-PAGE and Western blots [27, 93].

1.3.10. PCV Epidemiology & measuring antibodies

Serological surveys indicate that antibodies to PCV2 are prevalent in swine herds, with or without PMWS, globally [72, 83, 87, 94-101]. There are reports indicating that the prevalence of

PCV2 antibodies within a herd could be higher in PMWS-affected herds compared to non-affected herds [83, 102], or be equally high in both groups [95, 98]. The high prevalence of PCV2 antibodies compared to PMWS incidence indicates that PCV2 is well adapted to its host and subclinical infection prevails. IPMA [83, 95] and indirect immunofluorescence assay (IFA) [103] demonstrated a greater prevalence of antibodies to PCV2 antibodies compared to PCV1. This observation is in accord with that of a lower occurrence of PCV1 tissue DNA (6.4%) compared to PCV2 (14.7%) detected by PCR in healthy pigs [26]. Further, a larger proportion of PCV1 seropositive pigs had PCV2 antigens in their tissues compared to pigs without containing PCV2 antigens [83], suggesting that PCV2 infection possibly can induce cross reactive antibodies to PCV1. Pigs containing PCV1 antibodies but negative for PCV2 antibodies were reported, although in low prevalence [103]. Competitive enzyme-linked immunosorbent assay (cELISA) using monoclonal antibodies (mAb) were developed to measure anti-PCV2 antibodies [79, 94]. In addition, indirect ELISA (iELISA) methods were developed to measure anti-PCV2 antibodies using either recombinant PCV2-Cap protein [99, 102, 104-106] or PCV2 infected cell lysate [107] as coating antigens. Sera from humans and a range of animal species including cattle, sheep, pigs, goats, chickens, turkeys, rabbits and mice tested by IFA did not contain anti-PCV antibodies [72]. In a similar experiment, antibodies reactive to purified PCV1 antigens were detected using IFA and ELISA in human, cattle and mice but these results were suggested to result from cross reactive antibodies caused by related species specific viruses [93].

1.3.11. Post-weaning multisystemic wasting syndrome (PMWS)

PMWS, one of the PCV2 associated diseases (PCVAD), was first recognized by a group of scientists at the University of Saskatchewan in several high health herds in Western Canada in 1998 [31, 108, 109] and subsequently reported worldwide [17, 26, 31-36, 50, 52, 100, 110-112]. PMWS causes considerable economic losses to the swine industry [113]. In recent years, there was an upsurge in severe outbreaks in Europe and North America [52, 53, 59]. The most prominent clinical signs are wasting, dyspnoea, enlarged lymph nodes, diarrhoea, pallor, and jaundice. The clinical manifestations are usually restricted to the post-weaned aged groups, between 7 and 15 weeks, particularly affecting pigs in late nursery and early grower stages [114, 115]. Serosurveys indicate virtually all commercial herds are infected, although only a small

proportion of pigs develop the disease [98, 99, 103, 115]. Experimentally reproduced [28, 37, 39, 40, 42, 43] or naturally occurring PMWS cases [116-119] have increased severity of lesions and higher mortality rates when pigs are concurrently infected with other common pathogens of pigs such as porcine parvo virus (PPV), porcine reproductive and respiratory syndrome virus (PRRSV), swine influenza virus and *Mycoplasma hypopneumoniae*. Mortality rates up to 30% were reported on farms experiencing such multiple infections [120]. Death of affected animals (case fatality rate) can be as high as 70-80% [121].

Since PCV2 was first isolated from the Canadian index farm, several studies have attempted to reproduce the clinical disease by inoculating PCV2 alone or in combination with other co-pathogens in order to fulfil Koch's postulates in different experimental pig models including conventional pigs, with or without colostrum deprivation, inoculated with PCV2 and PRRSV [42, 43], conventional pigs inoculated with PCV2 alone [40, 45, 46] or in combination with PPV [40], SPF pigs inoculated with PCV2 tissue homogenate [41] or cloned PCV2 DNA [122, 123], gnotobiotic pigs inoculated with PCV2 in combination with PPV [28, 39], immune stimulation with KLH/ICFA [38] or immune suppression [124]. These experiments had varying levels of success in reproducing the disease. Discrepancies were mainly attributed to the differences in the pig model and the challenge inocula/method used. However, the gnotobiotic pig model with immune stimulation produced clinical disease in all inoculated pigs and fulfilled Koch's postulates [38]. Production of disease with inocula containing cloned PCV2 DNA supported the latter finding and confirms PCV2 is the primary/essential cause of PMWS. Therefore, the role of co-pathogens is considered to be that of a potentiating factor [125]. PCV2 has also been incriminated in other diseases including porcine dermatopathy nephropathy syndrome (PDNS) [126-128], porcine respiratory disease complex (PRDC) [129], reproductive disorders [130] and proliferative and necrotizing pneumonia [131].

Diagnosis of PMWS is based on combination of number of criteria i.e., i. typical clinical signs, ii. characteristic microscopic lesions, and iii. demonstration of viral antigens and/or nucleic acid associated with histological lesions in affected pigs [75, 116]. None of these criteria separately or demonstration of antibodies is indicative of the PMWS, since healthy pigs can harbour PCV2 in their tissues and have serum antibodies.

1.3.12. PCV2 pathogenesis

PCV2 persists as a subclinical infection in most commercial pig herds [83, 95, 98, 99] and the virus maintains a stable relationship with the host in spite of seroconversion [132]. Both healthy and diseased pigs shed virus/viral DNA in excretions and secretions [92] contaminating the premises. The virus is stable and persists in the environment for prolonged periods of time, resulting in the exposure of newborn piglets [72, 75, 121]. The mode of infection is widely believed to be through the oronasal route [121]. Maternal antibodies have partial protective capacity [133]. However, these decay after weaning, reaching minimum levels by 9-10 weeks which coincides with viremia (viral DNA by PCR), viral excretion and elevation of PCV2 antibodies [87, 92, 134]. Although not proven, epithelial cells are considered a possible primary source for viral replication [63] as evidenced by the presence of viral antigens and/or DNA in respiratory, intestinal and renal epithelia of infected pigs, and hepatocytes as well as in cardiomyocytes of foetuses [80, 124, 135]. The general consensus is that virus laden macrophages and DCs serve as the mode of systemic dissemination [63]. PMWS affected pigs have elevated PCV2 antibody titers but lack virus neutralizing antibodies (VNA) and cell-mediated immunity (CMI) [134, 136-138], which explain the reasons for uncontrolled viremia leading to PMWS development.

The typical microscopic lesions observed in natural PMWS-affected pigs include extensive and severe depletion of both B and T cell regions of lymphoid tissues and their replacement by infiltrating viral laden histiocytes, macrophages and follicular dendritic cells (FDC), the formation of giant cells in lymphoid organs, thymic atrophy, granulomatous hepatitis with hepatocyte necrosis, interstitial pneumonia, interstitial granulomatous enteritis and granulomatous nephritis [39, 125]. In contrast, in subclinical infections in gnotobiotic and conventional pigs there is increased cellularity and germinal centre development in lymph nodes and lympho-histiocytic infiltration in liver, heart and kidney [63, 125]. The transition from subclinical infection to clinical disease is accompanied by the accumulation of large amounts of viral antigen in infiltrating mononuclear cells and the disappearance of germinal centres [63]. However, in both *in vitro* [62, 139] and animal experiments [63] there was no evidence for PCV2 replication in macrophage and DCs. It is likely that progressive accumulation of viral antigens in these cells occurs as the result of phagocytosis [63]. Uptake of viral antigens can also be

enhanced by non-neutralizing antibodies [140]. Infectivity of the virus persists for a long period of time [61, 62] as a consequence to its ability to inhibit cellular endosomal maturation pathway [61]. The viral load in lymphoid tissues is positively correlated with the severity of lesions and clinical disease [28, 124, 125]. A similar correlation exists between viral load in the inguinal lymph node and the absence of PCV2 neutralizing antibody and CMI response to the virus [134, 137]. Severe lesions in PMWS are associated with co-infections such as PRRSV, swine influenza and *Mycoplasma hypopneumoniae* [120].

The hemogram of PMWS-affected conventional pigs shows lymphopenia, neutrophilia and monocytosis, while the total leukocyte number remains within normal range. Lymphocyte and neutrophil counts are 30-40% and 60-70% respectively, almost the reverse of a normal hemogram. Both T and B lymphocyte counts are significantly depressed [138, 141-144]. However, affected pigs are not under general immune suppression as demonstrated by the persistence of PCV2 antibody titers [138] and the ability to mount humoral responses to unrelated antigens such as KLH in infected gnotobiotic pigs [124].

Analysis of cytokine mRNAs expression pattern in a range of lymph tissues provided a likely explanation for the altered immune responses in PMWS affected pigs [138]. Darwich et al (2003) showed significantly reduced expression of mRNA for IL-2, IL-4, IL-10, IL-12 and IFN-γ in inguinal lymph node and spleen, and increased IL-10 mRNA in the thymus compared to healthy pigs [138]. Similarly, peripheral blood mononuclear cells (PBMC) isolated either from PMWS affected pigs or healthy pigs exposed to PCV2 did not express IL-2, IFN-γ and IL-4 upon stimulation with either T cell mitogen (PHA) or staphylococcal enterotoxin B (SEB) super antigen [145], indicating a direct association between exposure to PCV2 and altered cytokine expression in lymph tissues. Further, plasmacytoid DC (natural interferon producing cells) incubated with PCV2 failed to respond to cytosine-phosphate-guanine dinucleotide (CpG) by upregulating MHC class II and CD80/86 costimulatory molecules and resulting in inhibition of TNF-α and IFN-α production required for myeloid DC (mDC) maturation, thereby affecting recognition of viral and bacterial antigens [139]. Virus neutralizing antibodies (VNA) are epitope specific [146]. VNA titer and their affinity depend upon multiple factors, including host genetics, nature of the viral epitope, duration of antigen exposure and the antibody isotype [147]. Impaired generation of VNA and CMI in PMWS affected pigs appears to be a consequence of

deregulation of immune functions by PCV2. The sequel to this is the enhancement of PCV2 replication and vulnerability to co-pathogens. This suggestion was supported by the finding that vaccinated pigs developed VNA and CMI, and abrogated PCV2 viremia and co-infections [60, 148, 149].

Altered cytokine expression and impaired DC function caused by PCV2 also suggests a possible mechanism for the depletion of lymphocytes in lymph tissues in PMWS affected pigs. Clonal deletion of T lymphocytes is described subsequent to prolonged and overwhelming antigen stimulation by virus laden macrophages and DCs in the absence of co-stimulatory signals, i.e., expression of cytokines as occurs in PMWS affected pigs [150, 151]. This possibility is more convincing since findings of *in vitro* [61] and most of animal experiments [28, 39, 63, 124], with a few exceptions [152, 153], are in agreement that lymphocytes do not permit PCV2 infection. Therefore, viral replication-associated damage to lymphocytes is unlikely. Further, the finding that lymphoid depletion was a result of decreased cell proliferation rather than increased apoptosis [154] strengthens the former explanation.

The mechanism of reproduction of PMWS in gnotobiotic pigs by immune stimulation [38] is likely mediated by induction of proliferation of cells that are minimally permissive to PCV2 [63]. This explanation is based on the finding that PCV2 replication is dependent on cellular expression of DNA polymerases during the S phase of the cell cycle [76]. Immune stimulation also generates non-specific antibodies which can enhance viral uptake by macrophages and DCs [140], leading to increased lymphoid tissue viral load resulting in impaired immune functions. Antibody mediated enhancement of cellular uptake of viruses, thereby facilitating their replication and leading to augmentation of disease severity was described in the pathogenesis of diseases such as PRRSV, Dengue Fever and importantly in PDNS [140, 155-157]. The increased severity of lesions and clinical outcome because of co-pathogens [37, 39, 75, 118] can also be explained by both of these mechanisms, i.e., induction of proliferation of viral permissive cells and generation of non-specific antibodies. In summary, the principal characteristics of PCV2 pathogenesis are lymphoid depletion and impaired generation of VNA and CMI, leading to uncontrolled viral replication.

1.3.13. Likely mechanism of wasting in PMWS affected pigs

Wasting is a characteristic clinical feature of PMWS and affected pigs loose both muscle mass and fat deposits [158, 159]. Multiple mechanisms may be involved in any wasting syndrome. However, all the mechanisms collectively lead to a negative energy balance either by lower energy intake, lower feed conversion rate [158], as reported in PMWS, or increased metabolism. One of the possible mechanisms of loss of muscle mass and fat deposits is through systemic elaboration of proinflammatory cytokines, as observed in natural PMWS affected pigs [160].

Pro-inflammatory cytokines such as TNF-α and IL-1 act on the hypothalamus and cause fever and anorexia [161]. Anorexia causes less food intake while fever increases heart and metabolic rates. Pro-inflammatory cytokines induce lipolysis and oxidation of fat deposits, and stimulate the release of cortisol and catecholamines from the adrenal gland which increases the metabolic rate [162], resulting in increased energy consumption. Affected pigs suffer from jaundice and diarrhea [114] suggesting that impaired digestion and absorption of nutrients contributing to lower energy intake. Loss of muscle mass is attributed to increased protein catabolism, as evidenced by elevated blood urea levels in affected pigs [163], or to impaired synthesis mediated by pro-inflammatory cytokines such as TNF-α, IL-1β, IL-2, IFN-γ and IL-6 [164-168].

The mechanism of action of the pro-inflammatory cytokines is through inhibition of an anabolic hormone, insulin-like growth factor 1 (IGF-1). This hormone is required in protein synthesis in skeletal muscle. Pro-inflammatory cytokines can inhibit the synthesis of this hormone at the transcriptional level or through the growth hormone axis by inhibiting stimulatory receptors on hepatocytes for growth hormone (GH) [167] . Elevated IGF binding protein-1 is also associated with a decrease in the rate of muscle mass reduction in wasting disease situations [167]. These cytokines can also exert direct effects on muscle protein synthesis by activating nuclear transcription factor (NF-κB). TNF-α and IFN-γ act synergistically to inhibit mRNA synthesis for myosin heavy chain. Cytokines also activate ubiquitin, a 76 aa proteolytic polypeptide responsible for muscle degradation [162]. In summary, one of the likely mechanisms of wasting in PMWS affected pigs is mediated by pro-inflammatory cytokines.

1.3.14. Economic impact of PCV2 infections

The economic losses attributed to PCV2 infections result from increased mortality and culling, low market value for stunted pigs, reduced production parameters including lower feed conversion and average daily weight gain, poor reproductive performance including reduced number of live born and weaned piglets per litter, weaned piglets/productive sow/year and weaning-to-next heat interval, and the treatment, labour and veterinary intervention costs [113, 120, 159, 169, 170].

1.3.15. PCV2 control strategies

Control strategies for PCVAD are two-fold: vaccination and improved management practices.

1.3.15.1. Improved management practices

An important element of combating PCV2 infection is minimizing on-farm infective and non-infective co-factors [108]. Co-pathogens such as PPV, PRRSV, *M. hypopneumoniae* and swine influenza exacerbate the severity of PCV2 induced lesions and cause explosive outbreaks, therefore, control of these infections is a primary focus in minimizing losses due to PCVAD [75, 115, 117, 118, 120].

The major goals include minimizing pig-to-pig contact and improved hygiene and nutrition [113, 171-173]. Pig-to-pig direct contact is identified as a potential means of viral spread in a herd while indirect contact through needles, surgical instruments, manure or people are documented as other possible means of spreading viruses [173]. Establishing solid partitions between pens and adopting all-in,all-out systems across the farm are recommended to reduce pig-to-pig contact [173]. The quarantine of purchased pigs is aimed at preventing the introduction of new infections. Thorough cleaning and disinfection procedures using an effective virocide, eg., Virkon (ANTEC® BIOSENTRY® Windham Road Chilton Industrial Estate Sudbury Suffolk, CO19 2XD United Kingdom), and good hygiene measures including regular changing of boots and clothes, stopping teeth clipping, prompt removal of sick and dead pigs, proper disposal of dead pigs are recommended to curb the spread of infection within herds/farms. Good colostrum management, i.e., adequate colostrums intake within 24 h of farrowing,

increased access to nutritious feed and disinfected water, improved temperature, ventilation and air quality and reduced pen size are measures aimed at improving health status and reducing the stress imposed upon the pigs.

1.3.15.2. PCV2 vaccines

Currently, there are four commercial PCV2 vaccines available in Canada [96]; Circovac® by Merial Canada Inc. (20000 Clark Graham, Baie d'Urfé Québec, H9X 4B6) containing the whole virus, Suvaxyn® PCV2 One Dose by Fort Dodge Animal Health (P.O. Box 25945Overland Park, KS, USA) [174] containing PCV1 engineered to express PCV2-Cap replacing its own capsid, Circumvent^{TM} PCV by Intervet Canada Corp (16750 route Transcanadienne Kirkland, Quebec, H9H 4M7) [60] and CircoFLEX$^{®}$ by Boehringer Ingelheim Canada Ltd. (5180 South Service Road, Burlington, Ontario, L7L 5H4) [149] containing PCV2-Cap expressed through a Baculovirus vector. All these vaccines contain inactivated PCV2-Cap as the vaccine antigen and, an adjuvant was incorporated except for Suvaxyn®. Circovac® is intended for sow vaccination; the primary vaccination is given intramuscularly at least 2 weeks before mating and the secondary at least 2-4 weeks before farrowing. Suvaxyn® PCV2 One Dose is administered intramuscularly once to healthy pigs 4 weeks and older. Circumvent^{TM} PCV is recommended to be administered twice intramuscularly, 3 weeks apart to healthy pigs 3 weeks and older. CircoFLEX$^{®}$ is a single dose vaccine administered intramuscularly to healthy pigs 3 weeks and older [96].

There are two recommended vaccination strategies. Circovac® is recommended to be given to sows, relying on the protective effects of passive transfer of maternal immunity to young piglets. The other three vaccines target piglets; primary immunization is recommended at 3-4 weeks of age to induce active immune responses prior to the anticipated expected high risk period. In one study [175], mortality rate up to finisher age (~19-22 weeks) were compared among four groups of pigs i.e., i. immunized through sow vaccination (passive immunization) ii.& iii immunized at young age (active immunization) with two different PCV2 vaccines designated A & B and iv. non-immunized controls. There was no difference in mortality rate between non-vaccinated pigs (10.7%) compared to pigs born to vaccinated sows (10.4%). However, piglets vaccinated at a young age had significantly lower mortality 3.9% and 3.1 % for vaccine A and B, respectively, favouring piglet vaccination over sow vaccination. PCV2

vaccines were shown to induce both humoral and cellular immunity and, thereby, reduce PCV2 viremia, co-infections, morbidity and mortality rates, and improve production and reproductive performance [60, 120, 148, 149, 158, 159, 169, 170, 176-181].

1.4. BACTERIOPHAGES IN GENERAL

Bacteriophages are viruses that infect bacteria, and are the most abundant organisms on earth, totalling ~10^{31} [182]. They are ubiquitous in all natural environments and present in human and animal skin and in intestinal tracts [183, 184]. Bacteriophages constitute the order of Caudovirales, 96% of which are tailed, and are classified into 10 families based on their morphology and genome: single-stranded (ss) versus double stranded (ds), DNA versus RNA [183, 185]. The size of the phage genome can vary from 4-600 kb, carrying genes coding for DNA transcription, replication, packaging, lysis and structural proteins including the head, tail and tail fibres [185]. There are two types of bacteriophages: virulent and temperate. Bacteriophages, through elements on the capsid, bind to specific receptors on the target bacterium, and inject their genome into the host cell. For virulent phages, the next steps of the life cycle are the initiation of viral replication followed by the production of progeny viruses. The life cycle of a temperate phage, such as lambda infecting *E. coli.*, can be through one of two pathways: the viral genome can either integrate into the host genome (lysogeny), or undergo a replication cycle releasing progeny viruses by lysis of the host cell [186]. In contrast, filamentous phages do not lyse the bacterium but progeny viruses are released by extrusion from the cell membrane. Bacteriophages are incapable of binding and infecting organisms more complex than bacteria, because of differences in cell-surface molecules and intracellular machinery essential for phage replication [183]. Therefore, they do not replicate in eukaryotes and have been shown to be safe following oral administration in humans [187].

Bacteriophage lambda is a temperate phage infecting *E. coli* that contains linear ds DNA genome of 48.5 kb with single stranded termini of 12 nt (cohesive termini, *cos* termini) [188]. The lambda head is icosahedral, 55 nm in diameter and holds a 180 nm long, flexible, helical tail made of protein V arranged as a stacked disc tube terminating at the tail fibre made of J protein [189, 190]. The lambda genome is organized into functionally related clusters i.e., left-hand region: genes from Nu1 through J which control morphogenesis, central region: J through *gam* involved in gene regulation, establishment and maintenance of lysogeny and genetic

recombination, and right-hand region: *gam* through *Rz* which are essential in viral replication and lysis of the host cell [188]. Two clusters of genes, seven genes from A-F and 11 genes from Z-J, on the left-hand region govern the synthesis of the head and tail, respectively [191]. Lambda attaches to the bacterium using a specific receptor (LamB) and releases its DNA into the cell. *Cos* termini are base paired and formed closed circular DNA which serves as the template for transcription. DNA replication is initiated bidirectionally and then switches to rolling circle. The lytic cycle is completed in about 45 minutes and releases ~100 virus particles per infected cell [188].

The assembly of lambda particles is a multi-step process governed by both phage and host cell coded factors. In this sequential process, specificities for the assembly components arise by conformational changes [192]. If this process becomes defective, as in the generation of a mutant phage, all gene products, except one, are synthesized and, therefore, either a stable precursor lambda particle or a visibly different abortive particle is formed. In the normal process, morphogenesis begins in the middle of the latent period while the precursor molecules are still being synthesized. At the initial step, the head begins to assemble under the control of 10 phage coded genes including genes A through F and Nu3; E and D are the two major structural proteins on the fully formed head [190]. First, a connector, a ring shaped structure is formed with 12 monomers of B which interact with C and serve as the nucleation site for polymerization of E to form the prohead [193, 194]. Minor proteins B, C and Nu3 (sacffold protein) guide E in the proper assembly of the capsid protein shell, their absence leads to formation of a long tubular polyhead [190]. The prohead becomes competent in encapsidation of DNA after cleavage of the minor proteins. Replicating DNA made up of tandem repeats of mature ds DNA is packaged into the preformed prohead until it reaches its full capacity and cleaves from the extra long DNA molecule [190]. Protein A with its nuclease activity is responsible for this cleavage following the recognition of protruding 5′ single strand (cos terminal) of the encapsulated DNA. Proper assembly of the prohead is vital to produce progeny virus with a complete genome with double *cos* ends. Mutants with a defective prohead lead to accumulation of replicating DNA without completion of the packaging step [190]. During DNA packaging, the head expands ~20% [193, 195] and protein D is incorporated into the underlying prohead through its first 15 aa to stabilize the head [188], enabling packaging of full-length DNA. D-defective phages encapsidate only up to ~80% of complete genome [195], indicating that protein D plays a role in lambda

morphogenesis [188]. The mature lambda head consists of two major proteins, E and D, with 415 and 405-420 copies, respectively. Protein E has a MW of 37.5 kDa [196] and is arranged in hexameric and pentameric capsomeres while D with MW ~11 kDa is folded into trimers [188] on each of 20 faces of lambda head. To complete morphogenesis, W and F1 proteins prepare the head to join with the tail [190]. The tail binds to a corner of the head that had altered its symmetry being the site of either initiation, termination of head assembly or to which DNA ends are directed [190]. However, this binding process occurs in a manner ensuring release of phage DNA through the tail in the subsequent infection cycle.

Bacteriophages were studied mainly to understand molecular genetics of higher organisms [189, 197]. In addition to use of bacteriophage as therapeutic agents, knowledge of bacteriophage genetics paved the way to develop biologicals such as vaccines, gene therapy vehicles and diagnostic reagents. The discovery of phage encoded toxin genes, along with the analysis of bacterial genome sequence data, led to understanding the role bacteriophages play as important vehicles for horizontal transfer of virulent genes and, thereby, evolution and virulence of many pathogenic bacteria [198]. One category of bacterial virulence factors acquired through bacteriophages includes exotoxin genes such as diphtheria toxin in *Corynebacterium diptheriae* by β-phage, Shiga toxins in enterohemorrhagic *E. coli* O157:H7 by lambda phage and cholera toxin in *Vibrio cholera* by a filamentous phage [198-206]. Other types of virulence factors transferred through phages are effector proteins involved in invasion, such as type III secretary system in Salmonellae, enzymes such as hyaluronidase in *Streptococci pyogenes*, serum/phagocyte and antibiotic resistance factors and components necessary for bacterial adhesion and colonization in *Streptococci mitis*, causative agent for infective endocarditis [198, 201].

Phage-mediated gene transfer occurs via two mechanisms: transduction and lysogenic conversion. It is estimated that such gene transfer events occur at a rate of up to 2×10^{16} per second [198]. In transduction, phages infecting a bacterium (transducing phages) pick up a fragment of host genome, package it into progeny virus and transfer it into another bacterium. In lysogenic conversion, certain temperate phages such as lambda, Mu and p2 phages incorporate their genome into the bacterial chromosome. In such an event, the bacterium and the incorporated phage genome is termed “lysogen” and “prophage” respectively. Gene transfer by

these mechanisms can occur between bacteria within the same or different species [198, 199]. Lysogenic conversion allows the bacterium to be protected from lysis by a subsequent homologous phage infection (immunity) and, thereby, gains fitness in the environment compared to nonlysogenic counterparts [198]. Prophage is relatively stable and carries over to each daughter cell when the bacterium multiplies. However, certain stimuli such as UV radiation and antibiotics that damage bacterial DNA can induce prophage to initiate viral replication and produce progeny viruses. Certain bacterial pathogens exist in a less virulent, stage until prophage induction, and become increasingly virulent, with production of virulent factors since expression of virulent genes is regulated as part of phage life cycle [201]. Released phage carrying virulent genes can subsequently infect other resident bacteria leading to amplification of the pathogenic effect. The spread of certain diseases, such as cholera, is reported to be due to the dissemination of toxigenic phage rather than bacterium itself [205].

The ability of lambda phage to switch between lytic and lysogenic pathways is reported to be partly attributed to the manner of its genomic organization, which ensures a typical cascade of their expression in the order of early, delayed early, and late genes [207]. Repression of early phage promoter genes prevents expression of late lytic genes and arrests in lysogenic state. This repression is carried out by the expression of CI protein which binds to upstream phage regulatory genes and prevents prophage from entering into the lytic pathway. Conversely, inactivation of CI triggers the expression of genes necessary for producing progeny virus and lysis of host cell [207]. Therefore, lambda mutant containing temperature sensitive CI can be used to regulate expression of phage proteins by thermal induction. Lysogen can also spontaneously loose the prophage in a process referred to as “curing”.

In order to use bacteriophages in biotechnology applications, the kinetics and fate of bacteriophages in biological systems were studied in *in vitro* experiments [208, 209] and in laboratory animals inoculated through intraperitonial, intramuscular and intravenous routes [210, 211]. A study using germ-free mice revealed the spleen had the highest phage titer at 3 hpi, irrespective of route of administration, and that titer was maintained for a period up to 7 days although phage titers declined to zero in blood by 48 hpi [208]. Bacteriophages, being particulate antigen, were reported to be rapidly removed by phagocytes in organs of the reticuloendothelial system (RES) including liver and spleen [208, 210]. Persistence of viable phages for long periods

of time in spleen was suggested to be due to spleen's ability to capture antigen through a non-destructive process, ensuring a source of viable phage for immune stimulation [208] and for therapeutic effect [212]. The advantages of using bacteriophages in therapeutic and biotechnology applications are reported to include the feasibility of producing them rapidly and economically in large-scale, host specificity, safety, stability in a wide range of pH and temperatures, ability to target antigen presenting cells (APC) and their natural adjuvant activity thereby inducing both cellular and humoral immunity [210, 213-216].

1.5. BACTERIOPHAGE TECHNOLOGY APPLICATIONS IN VETERINARY MEDICINE

1.5.1. PHAGE THERAPY

With the increase in incidents of emerging antibiotic resistant pathogenic bacteria, there is a renewed interest in the use of bacteriophages as a therapeutic means in livestock, aquaculture and agriculture industries [186, 212, 217-226]. Important and successful veterinary applications include controlling diarrhoea due to *E. coli* in cattle and pigs and minimizing shedding of food borne pathogens such as *E. coli* O157:H7, *Campylobacter* and *Salmonella* by cattle and poultry. Advantages of phage therapy over the use of antibiotics include host specificity, relatively low cost and the phage being a viable agent replicating in the host and, therefore, exert an amplifying therapeutic effect [222]. Examples of therapeutic phage applications in veterinary medicine are described below.

Cattle:

Young calves aged 6-12 hr separately infected with six different nonlysogenic enteropathogenic K^+ *E. coli* strains at a dose of 10^9 per animal were protected from otherwise developing fatal diarrhoea by oral administration of 10^5 previously characterized bacteriophages. Protection was observed either with a dose of 10^5 or 10^2 phage treatment given 6 hr or 10 min prior to *E. coli* infection, respectively. Calves had significantly reduced bacterial counts in the faeces. On testing practical and convenient methods of phage treatment, protection was shown when calves were allowed to feed on litter sprayed with an aqueous solution of phage preparation 3 hr prior to *E. coli* infection. Similarly, six calves orally infected with a mixture of *E. coli* strains were protected from death by exposing them to a cocktail containing six virulent phages each specific for the respective bacterium [226]. In a similar study, five 6-month old

calves experimentally infected with *E. coli* O157:H7 had 1.5 log reduction in fecal bacterial shedding following rectal administration of a mixture of two lytic bacteriophages at a dose of ~ 8 X 10^{10} pfu per animal per day combined with oral treatment by phage added to the drinking water to a final concentration of 5 x 10^5 pfu/ml for 4 days [217].

Purified *Staphylococcus aureus* endolysin expressed in *E. coli* using a bacteriophage gene construct showed lysis of the bacterium *in vito* [224]. To test the efficacy of bacteriophage treatment in mastitis, healthy cows with subclinical *Staphylococcus aureus* mastitis were administered an intramammary infusion of 1.25 x 10^{11} pfu virulent phage per quarter per day for 5 days. Milk samples collected weekly for 4 weeks after treatment were tested for *S. aureus* by culture. Cure, defined as negative bacterial isolation at any point of sampling, was achieved in only 16.7% of treated quarters and was not significantly different from the controls. The lack of therapeutic effect was attributed to inactivation of phage by influx of inflammatory cells and inhibition of phage activity by milk whey [221].

Pigs:

Three-week old weaned pigs treated with a cocktail of six different lytic phages at10^{10} pfu/animal following oral inoculation with ~10^{10} pfu enterotoxigenic *E. coli* (ETEC) O149:H10:F4 were protected from diarrhoea and associated weight loss. Similarly, infected pigs treated with a mixture of two phages, 3 times at 6h intervals 24 h after developing diarrhoea, had a significant improvement in weight change and decreased severity and duration of diarrhoea indicating phages can effectively be used for both preventive and therapeutic purposes [219].

Poultry:

Three-week old SPF chickens with septicaemia and meningitis induced by intramuscular or intracranial administered *E. coli* O18:K1:H7 were protected when treated intramuscularly with 10^8 pfu of lytic bactriophage simultaneously or 8 h prior to infection [212]. In a similar study, 10-day old *Campylobacter* and *Salmonella* free broiler chickens experimentally infected with *C. jejuni* were treated with host specific bacteriophage orally at $10^{9\text{-}10}$ pfu per bird per day for 10 days until 4 days prior (preventive purpose) to bacterial challenge or for 5 days starting at 6 days after bacterial inoculation (therapeutic purpose). Caeca collected at 3-day intervals following phage therapy revealed success of both preventive and therapeutic treatment as bacterial colonization was reduced by 1 log over a period of 30 days compared to controls [220].

Although, there are successful therapeutic applications of bacteriophages, phage therapy is not yet a widely accepted method of treatment in medical and veterinary sciences. The reasons attributed to this situation include unreliable and inconsistent early phage therapy trials, lack of control studies published in peer reviewed journals, difficulties in maintaining viability of phages in biological systems due to phagocytosis, the presence of inhibitory substance such as pus and necrotic materials at the target site, the potential for endotoxic shock as a result of releasing large quantities of endotoxins from lysed gram-negative bacteria and the difficulties obtaining intellectual property rights [214, 224].

1.5.2. BACTERIOPHAGE DISPLAY SYSTEMS

Bacteriophage display is a versatile technique enabling the display of a wide variety of foreign peptides for multiple applications including antibody engineering and discovery, protein engineering technologies such as random and site directed mutagenesis, for the detection of biological threat agents, gene therapy, drug design, chemotherapy and for the development of diagnostic reagents and vaccines [188, 189]. Three bacteriophage display systems including filamentous phages [227-229], T4 [230] and lambda bacteriophages [5] have been used to develop vaccines. In the filamentous phage M13 display system, major coat protein (pVlll, 2700 copies) and minor coat protein (plll, 5 copies) are most widely used as fusion partners. Filamentous phages do not lyse the host cell. Hence, peptides intended to display must traverse across the host cell membrane which causes difficulties in displaying some foreign peptides. Further, display density i.e., number of peptides that are displayed per phage particle, is very low in this system and the size of the peptide is limited to six to eight amino acids, as larger peptides interfered with phage assembly [189]. These difficulties are avoided by using lytic phages such as T4 and lambda. Small outer capsid protein (SOC, 810 copies) and highly immunogenic outer capsid protein (HOC, 155 copies) are used in the T4 display system. Both HOC and SOC proteins are easily accessible and nonessential for phage morphogenesis, viability or infection [230, 231]. Phage lambda is reported to be a superior display system compared to filamentous and T4 bacteriophages in terms of variety, size and number of peptide copies displayable per particle (display density), without affecting phage morphogenesis [188, 197, 232-237].

High display density is critical in certain applications such as identification of ligands binding to specific antibodies to develop diagnostic assays and therapeutic vaccines for diseases

such as cancer, autoimmune diseases and age related conditions where antibody titers are generally very low, and for detecting biological threat agents where efficiency of detecting even few organisms is vital. For drug and gene delivery and especially for vaccine delivery vehicles it is preferable if the phage is entirely covered with immunogenic peptides [189]. Initially, peptides were fused to lambda major tail protein V [236]. This display system yielded a low number of fused molecules per phage particle [188]. Later, a high level of expression was achieved by fusing peptides to lambda head D protein [232]. Both the N and C termini of the protein D are accessible for fusion peptides since they are not at the trimmer interaction face and do not interact with protein E [188]. A higher display density was obtained by fusing peptides to C-terminal (88.4%) compared to N-terminal (~50%) [238].

1.5. 3. BACTERIOPHAGE VACCINES

1.5.3.1. Phage display vaccines

In an *in vitro* experiment to demonstrate filamentous bacteriophage as an antigen delivery system, antigen presenting cells pulsed with phage displaying a HIV-1 T-cell epitope fused at the N-terminus of pVIII major coat protein demonstrated antigen specific T cell proliferation. Sequence analysis of purified phage particles revealed only 10% of the pVIII copies available for fusing peptides was actually used up in this experiment [239].

Mice orally administered filamentous M13 phage displaying mimotopes, i.e., peptides that mimic specific ligands of human hepatitis B surface antigen (HBsAg), at a dose of 10^{12} particles per animal had an immune response only to phage coat proteins, without any measurable antigen-specific response by ELISA. The failed response to the target antigen was attributed to either inactivation by acidity in the stomach or to an inadequate amount of displayed peptides on the phage particles [227]. In contrast, mice immunized intraperitonially three times at 2 weeks intervals, with 1 mg of purified recombinant filamentous bacteriophage displaying protective epitopes of human respiratory syncytial virus (HRSV) showed antigen specific immune response and conferred complete protection following a lethal challenge dose [228].

Four types of recombinant filamentous M13 phages, each displaying one of four protective epitopes of *Taenia solium* fused to pIII protein were produced in bulk, inactivated and sterilized by autoclaving 30 min at 121 ^{o}C. In a placebo-control vaccine experiment, a phage preparation constituted with a cocktail of 10^{12} particles from each display product was

administered subcutaneously twice at monthly intervals to 3-4 month old piglets raised under field conditions in a porcine cysticercosis endemic area in Mexico. Pigs were examined for lesions of tongue cysticercosis 3-5 months after vaccination or for the presence of parasites in internal organs at slaughter. Tongue lesions were fewer in vaccinated pigs (3.9%) compared to the control group (14.2%), demonstrating 70% vaccine efficacy. The vaccine also reduced the number of infected pigs and their parasitic load resulting in a decrease in disease prevalence by 54.2% [229].

Recombinant T4 phage displaying foot-and-mouth disease virus (FMDV) full length capsid precursor polyproteins, namely P1 and proteinase 3C fused to SOC protein, was produced by inserting genes coding for P1 and 3C separately into the phage genome by homologous recombination; T4 phage infecting *E. coli* transformed with shuttle plasmid containing P1 or C3 cDNAs. Mice dually immunized with four doses of recombinant phages at 2 week intervals by intraperitonial (10^9 particles per mouse) or oral routes (10^{7-8} particles per mouse) showed 100% protection following a lethal dose of FMDV [230]. In a similar study, both SOC and HOC proteins of T4 phage were fused with ProA from *Neisseria meningitidis* individually or dually on a single phage particle. Mice immunized with these recombinant peptides showed seroconversion to respective peptides with higher titers against T4proA-Hoc compared to T4proA-Soc [231], proving HOC a better fusion partner compared to SOC in terms of inducing immunity.

1.5.3.2. Phage DNA vaccines

Hepatitis B surface antigen and eukaryotic promoter cloned into bacteriophage lambda, λ-HBsAg was injected four times into rabbits intramuscularly at a dose of 4×10^{10} particles per animal at 0, 9, 15 and 27 weeks. An anti-HBsAg immune response was shown in test animals compared to controls receiving recombinant HBsAg protein [240].

Although, there are number of requirements to be fulfilled prior to phage vaccine becoming widely practiced, one important issue to pharmaceutical companies is obtaining intellectual property rights [214].

1.5.4. BACTERIOPHAGE DIAGNOSTIC REAGENTS

A rapid and sensitive *E. coli* detection system was developed using biotinylated lambda phage. Temperature sensitive lambda lysogen was inserted with DNA coding for biotin binding

peptide (BBP) so that thermal induction resulted in generation of biotinylated lambda progeny virus particles displaying BBP fused to D protein (λgpD:bio). Biotinylation of BBP occurred naturally in the host bacterium using its own BirA enzyme. For the test, λgpD:bio were collected and added to a test sample that could contain *E. coli* together with streptavidin conjugated quantum dots (colloidal crystals a few nanometers in diameter). *E. coli* in the sample (target cells) were labelled with λgpD:bio-streptavidin QD complexes following an incubation step. Importantly, this technique used a mutant lambda phage that is incapable of lysing target *E. coli* cells. The sample was then analysed by flow and image-based cytometry to precisely detect and quantify the number of target *E. coli* cells. This technique is considered to be highly valuable in detecting slow growing or highly pathogenic bacteria such as *Mycobacterium* and *Bacillus anthracis* for which conventional diagnostic methods are either less efficient or require rigorous bio-safety regulations. This technology has the potential to detect different bacterial species simultaneously in a sample and is adaptable to field use due to broad environmental and storage stability of phage [241, 242].

A methodology was developed to detect viable *Bacillus anthracis* [243]. In this technique, gamma phage which replicates only in the presence of live *Bacillus anthracis* is detected using real-time PCR (RT-PCR) with 98% specificity. This technique is claimed to be quicker than the currently CDC approved standard protocol which requires isolation of colonies of *Bacillus anthracis* from an overnight culture and testing them for bacterium-specific capsular protein and plate lysis assay with gamma phage. Further, positive results in the new technique are indicative of the presence of viable *Bacillus anthracis* compared to its detection based on RT-PCR set to amplify bacterial DNA which detects nonviable bacteria as well.

In a similar approach, an assay was developed to detect *Mycobacterium avium* subsp. *paratuberculosis* in cow's milk [244]. Milk samples were incubated with the *Mycobacterium* phage and then plated on a lawn of amplifying host, *M. smegatis*. The resultant plaque was indicative of the presence of one of the four test phage sensitive *Mycobacterium* species. Positive samples are then tested by RT-PCR to confirm the presence of target species, *Mycobacterium avium* subsp. *paratuberculosis* using specific primers.

HYPOTHESIS

1. The parenteral administration of a prototype adjuvanted PCV2 vaccine can override maternally-derived PCV2 antibodies.
2. A lambda display PCV2 vaccine can overcome some of the drawbacks associated with commercial PCV2 vaccines.

OBJECTIVES

Maternal antibody interference to the induction of anti-PCV2 immune response is a constant concern when piglets are vaccinated at an early age against PCV2 associated diseases. My objective was to determine if the parenteral administration of a prototype, adjuvanted, PCV2 vaccine could override maternally-derived PCV2 antibodies, and induce active immunity in seropositive piglets.

There are few PCV2 vaccines available in Canada. All of them are killed vaccines likely containing varying amounts of spurious proteins that can cause adverse injection site reactions. The inactivation process involved in producing killed vaccines can inadverantly alter protective antigen epitopes and compromises vaccine efficacy. Production of these vaccines either by propagating PCV2 in cell cultures or expressing recombinant viral proteins *in vitro* is expensive. I wished to test a new lambda display PCV2 vaccine candidate that is very economical, highly immunogenic, and contains intact protective epitopes. First, I needed to determine if conventional pigs contain pre-existing antibodies against bacteriophage lambda, in order to ensure that the phage vaccine can be administered into pigs without the risk of suppressing the target-vaccine antigen-specific immune response. Secondly, I wished to prepare and characterize the vaccine candidate particles displaying immunodominant regions of PCV2Cap fused to lambda head protein D (LDP-D-CAP). Thirdly, it was essential to evaluate immunogenicity of the vaccine in conventional pigs. Finally, I wished to prepare and characterize two alternative lambda display preparations, LDP-D-FLAG and LDP-D-GFP and, displaying the FLAG tag and the green fluorescent protein respectively.

CHAPTER 2: EFFICACY OF PARENTERAL VACCINATION AGAINST PORCINE *CIRCOVIRUS* 2 (PCV2) IN SEROPOSITIVE PIGLETS

1. Introduction

Porcine *Circovirus* 2 (PCV2) are small, non-enveloped, single stranded DNA viruses [22]. PCV2 has been incriminated as a necessary cause for postweaning multi-systemic wasting syndrome (PMWS) [39]. This disease develops in piglets typically between 8-16 weeks of age, manifesting clinical signs including progressive emaciation, dyspnoea and enlarged superficial lymph nodes and results in a high case fatality rate [114]. PCV2 is also associated with a number of other diseases such as porcine dermatopathy nephropathy syndrome [126], reproductive disorders [130], proliferative and necrotizing pneumonia [131], and porcine respiratory disease complex [129]. Recently, a sharp increase in PCV2-associated deaths was reported in Canada [53, 245]. PCV2 is a very stable virus [72] and is ubiquitous in the swine population [114], making it very difficult to eradicate. Vaccination represents an attractive means to control the effects of this endemic infection and has been shown to be an effective strategy in controlling PCV2-associated diseases in some studies [60, 149, 246-248]. Several commercial PCV2 vaccines are available in North America. There are two recommended vaccination strategies: vaccinating sows and relying on passive transfer of maternal immunity, or immunizing young piglets to induce an active immune response. Effectiveness of sow vaccination can be complicated by several factors. First, maternally-derived PCV2 antibodies (MDAs) are generally short-lived (3-4 months) [249] and would not last until the PMWS high risk period between 7-15 weeks of age [114, 115]. Secondly, MDAs may confer only partial protection against such infections as PCV2 and rotavirus [133, 250]. Finally, MDAs possess virus neutralization capacity [251] but that alone may not be optimal or sufficient, since both humoral and cellular immunity may be essential in combating this viral infection. Vaccination at a young age could be used as an alternative, or together with sow vaccination; however, the efficacy of piglet vaccination may be negatively affected by MDAs for PCV2 in such an approach. To address the potential limiting effects of passive immunity, we investigated if parenteral administration of a prototype adjuvanted PCV2 vaccine to piglets at an early age could override MDAs for PCV2 and induce acquired immunity in young piglets.

2. Materials and methods

2.1. Formulation of the vaccine

PCV2 vaccine antigen was prepared according to previously reported methods [72, 78]. The amount of viral antigen and the total protein in the vaccine preparation was estimated by antigen capture ELISA [79, 84] and Bradford method [252], respectively. The LR4-incomplete oil-in-water adjuvant (Merial, Athens, GA, USA) was heated at 37^{o}C for 10 min followed by stirring for 1 min before it was cooled rapidly to 4^{o}C. The vaccine was diluted 1:1 with PBS (0.0036 M KCl, 0.0014 M KH_2PO_4, 0.136 M NaCl, 0.004 M Na_2HPO_4, pH 7.8) and maintained at 4^{o}C until dispensed over 1 min into an equal amount of adjuvant that was being stirred slowly at 4^{o}C. This vaccine formulation was stirred for an additional 1 min and stored at 4^{o}C overnight. Similarly adjuvanted PCV-free PK-15 (Dulac) cell lysate served as the control.

2.2. Preparation of challenge inocula

2.2.1. Spleen tissue homogenate

The viral inoculum for the initial challenge was prepared from a 10% (w/v) tissue homogenate of a spleen collected from an experimentally infected gnotobiotic pig. The homogenate was sonicated for 30 seconds at full power followed by centrifugation (BECKMAN COULTER, Avanti J-E, JA-17 rotor) at 12,000 rpm. Chloroform (5%, v/v) was added to the supernatant and mixed by constantly inverting the tube for 10 min at room temperature. The content was centrifuged at 2,000 rpm and the top layer removed from the chloroform. This was centrifuged (BECKMAN COULTER, Optima LE 80K, 70Ti rotor) at 30,000 rpm in order to pellet the virus. Pellets were suspended in PBS and filtered through a 0.2 μm filter.

2.2.2. Tissue culture lysate

PCV2 (Stoon 1010) was propagated in PCV-free PK-15 cells (Dulac) as described elsewhere [64, 76]. The medium covering the cell layer in tissue culture flasks (35ml/T175 flask) was removed, the cells were washed twice with PBS and the medium was replaced with minimum essential medium (Invitrogen, Carlsbad, CA, USA) with no fetal calf serum or antibiotics added. Cells were then frozen and thawed 3X, sonicated for 30 seconds at full power,

centrifuged at 10,000 rpm and the supernatant collected and mixed with commercially available palatable syrup prior to feeding the pigs.

2.3. Experimental design

Twenty 1-week old piglets from four different litters were purchased from a farm where PCV2 vaccination was not practiced, identified individually, randomized into vaccinate (10) and control (10) groups based on their weight. They were in four different farrowing crates at the farm at primary vaccination at week 1 of age. Vaccinates were administered a dose of 200 µl of inactivated adjuvanted PCV2 vaccine intradermally using an injector gun (Biojector 2000, Bioject Inc. USA). Each dose of vaccine contained PCV2 antigen equivalent to 4.75×10^4 50% tissue culture infective doses ($TCID_{50}$). Control pigs were injected with 200 µl of adjuvanted uninfected PK-15 cell lysate. At week 3 of age, they were brought to the WCVM animal care facility, group housed in a 15.4 square meter pen, fed a commercial medicated diet until week 10 of age and, thereafter, switched to nonmedicated pig grower ration. Water was supplied *ad libitum*. Vaccinates and controls were administered a booster dose of the vaccine and control preparation, respectively, at week 4 of age. Three weeks after the second treatment, both groups were challenged, initially by administering orally 2 ml of PCV2 inoculum, prepared from spleen tissue homogenate containing 1.6×10^5 $TCID_{50}$ of PCV2 per pig, followed by feeding inocula containing PCV2-infected PK-15 cell lysate and a fresh culture of *Campylobactor coli* and *Helicobactor cerdo* mixed with a commercially available syrup (Aunt Jemima Original syrup, Quaker Oats Company, Chicago, IL), delivering 3.6×10^3 $TCID_{50}$ of PCV2 per pig per day for next 3 days. Pigs were monitored visually for general health and clinical signs of PMWS during the experiment and euthanized by captive bolt at 3 weeks post-challenge. Gross lesions were assessed by a team of experts in this field who carried out the post-mortems. Inguinal, axillary, bronchial, mesenteric, gastric lymph nodes, thymus, tonsils, spleen, liver, ileum and bone marrow were collected in 10% buffered formalin for histopathology. Sera were collected prior to each immunization, challenge and euthanasia, i.e., at week 1, week 4, week 7, and week 10. The experimental protocol was approved by the University of Saskatchewan Animal Care and Supply committee (protocol number, 20050029).

2.4. Serology

2.4.1. Antigen capture ELISA

Antigen capture ELISA was performed as previously described [79, 84] to measure the amount of PCV2 antigens in the vaccine preparation and the challenge inocula. A flat bottom 96-well ELISA plate (Immunolon 4HBX, THERMO ELECTRON Corporation, milford, MA, USA) was coated with a monoclonal antibody (mAb, 2B1 F190) against PCV2 1002 isolate [79] in volumes of 100 µl per well at a dilution of 1:1500 in coating buffer (0.05M Sodium carbonate, pH 9.2). The plate was incubated overnight at 4^{o}C. The coating buffer was then removed and the plate was washed (4X) with PBS + 0.05% Tween$_{20}$ (PBST). The test samples and a standard PCV2 capture ELISA antigen (Stoon 1010 pool 28) with known $TCID_{50}$ were serially diluted (1:250, 1:500 etc) in PBST and 100 µL volumes of each dilution were added into duplicate wells. The plate was incubated 2h at 37^{o}C. After a similar washing step, 100µL per well of anti-PCV2 rabbit polyclonal antiserum was added at a dilution of 1:2500 in PBST and the plate was incubated for 1h at 37^{o}C. The plate was washed (4X) with PBST, 100µL per well of biotinylated goat anti-rabbit (Invitrogen, Carisbad, CA, USA) was added at a dilution of 1:4000 in PBST and the plate incubated at 37^{o}C for 1h. The plate was washed (4X) in PBST and 100 µl per well of Streptavidin-peroxidase conjugate (ABC reagent, Vector Laboratories Inc., Burlingame, CA, USA) diluted in PBST according to the manufacturer's recommendation was added and the plate was incubated at 37^{o}C for 30 min. 3.3', 5.5'-tetramethylbenzidine (TMB) substrate (KPL Inc., Gaithersburg, Maryland, USA) was added at 100 µl per well, after washing the plate (4X) with PBST. The reaction was stopped by adding 50 µl per well of 1M H_2SO_4 after 15 min of incubation at ambient temperature. The optical density of the solution in the wells of the plate was read at 450 nm. PCV2 antigen concentration in the test samples was calculated in terms of $TCID_{50}$ based on the standard curve plotted using respective concentrations in the standard PCV2 antigen dilutions.

2.4.2. Competitive ELISA (cELISA)

A competitive ELISA was employed to measure anti-PCV2 antibodies in pig sera as previously described [94]. The ELISA plate was coated with PCV2 antigen (PCV2 isolate # 48285) 100 µl per well at a dilution of 1:200 in a coating buffer (0.05 M Sodium carbonate, pH

9.2). The plate was incubated overnight at 4^{o}C. The coating buffer was then removed and the plates were washed (4X) with PBS + 0.05% Tween$_{20}$ (PBST). Each serum sample diluted at 1:125 in PBST was added in volumes of 50 µl per well in quadruplicate (two adjacent rows and columns of the plate per sample) and incubated at 37^{o}C for 45 min. Each plate included four wells each for blank (no serum, PBST only), negative and positive control sera. Without washing the plate, in alternative columns (2, 4, 6 etc.) monoclonal antibody (B12B6) was added to PCV2 isolate 48285 at 1:100 in PBST in volumes of 50 µl per well while to the remaining columns (1, 3, 5 etc.) only PBST in volumes of 50 µl per well. The plate was incubated at 37^{o}C for 30 min, washed 4X with PBST and horse radish peroxidise (HRP)-conjugated goat anti-mouse antiserum (Invitrogen, Carisbad, CA, USA) at a dilution of 1:5000 in PBST containing 5% (v/v) goat serum (Invitrogen, Carisbad, CA, USA) added in volumes of 100 µl per well. The plate was incubated at 37^{o}C for 1h and washed 4X with PBST before addition of TMB substrate (KPL Inc., USA) in volumes of 100 µl per well. Color development was stopped with 1M H_2SO_4 in volumes of 50 µl per well after incubation for 10 min at room temperature. The optical density of the substrate solution was read at 450 nm. Blank wells containing only the monoclonal antibody (mAb) without test sera represented a complete reaction (0% inhibition) while in wells containing both mAb and test sera containing PCV2 antibodies compete for the coated PCV2 antigen, hence, the reaction is inhibited. The percentage of inhibition was calculated for each sample as previously described [94].

2.5. Histology and Immunohistochemistry (IHC)

Inguinal and gastric lymph nodes were fixed in 10% buffered formalin, embedded in paraffin, sectioned at 5µm and rabbit anti-PCV2 polyclonal antisera used to detect PCV2 antigens by IHC [39]. H & E stained sections of left inguinal and gastric lymph nodes were examined for lymphoid depletion, lymphoid hyperplasia (follicle formation), germinal centre development, histiocytic hyperplasia and syncytial giant cell formation. The amount of PCV2 antigens, detected by IHC, were evaluated without knowledge of the identity of the sample on a scale of 0 to 3+; 0=negative, 1+=minimal, 2+=moderate, and 3+=Extensive.

2.6. Statistical analyses

The net change in cELISA values for individual pigs from primary vaccination to booster, challenge and 3 weeks post-challenge, respectively, as calculated by subtracting relevant individual animal cELISA values. The Wilcoxon Rank Sum test was used to test comparisons as data were not normally distributed. Comparisons with a *P*-value < 0.05 were considered significant. Statistical testing was done using a commercial software package (Statistix 8; Analytical Software, Tallahassee, Florida, USA).

3. Results

3.1. PCV2-specific antibody responses

Anti-PCV2 antibodies measured by cELISA in individual control (8) and vaccinated pigs (10) are presented in Figures 2.1 & 2.2, respectively. Though the vaccination trial began with ten pigs per group, one control pig was euthanized because of infectious arthritis and data for another control pig was not considered as it was mistakenly immunized at the booster vaccination. MDAs for PCV2 were not significantly different (> 80% inhibition in all pigs) between the two groups at 1 week of age. Antibody levels declined in control pigs until challenge at 7 weeks of age, whereupon antibodies increased in all piglets except for piglet # 14 (Fig. 2.1). Among vaccinated piglets, there were two patterns of change in PCV2 antibodies; five of 10 pigs (sub group A) maintained a higher level of PCV2 antibodies ($P = 0.008$; Range: 70-100% inhibition (Fig. 2.2) from week 1 to week 4 following primary vaccination compared to the remaining five pigs (sub group B) whose antibody levels decayed similarly to controls (Fig.2.1.). Only three of the pigs in sub group A (# 13, 28 and 30) maintained a high level of immunity following booster vaccination at 7 weeks of age and one of these pigs showed a decline in titer by week 10 (# 28). cELISA values for vaccinates as an overall group were not significantly different from controls at any time period. All but two immunized pigs (# 18 & 28) mounted an immune response to challenge (i.e., week 10, Fig. 2.1 & 2.2). Importantly, this response to challenge included 2/3 of the vaccinates (pig # 13 and 30) that maintained high cELISA titers (~80% inhibition) at 7 week of age.

Net decrease in cELISA value, compared after completion of vaccination (Week 1 to Week7) was less in vaccinates compared to controls ($P = 0.04$; Fig. 2.3).

3.2. Clinical signs and gross lesions

None of the controls or vaccinated pigs had clinical signs of PMWS during the experiment or gross lesions at post-mortem examination.

3.3. Histology and IHC

All the controls and vaccinates had microscopic lesions including lymphoid hyperplasia, germinal centre development and histiocytic hyperplasia (Table 2.1). However, no pigs had lymphoid depletion and syncytial giant cell formation, two characteristic microscopic lesions of clinical PMWS. By IHC staining, PCV2 antigens in left inguinal and gastric lymph nodes were detected in one and two of the eight controls, respectively (Table 2.2). The antigen staining intensity score was 1+ for all positive stains in a scale ranging from 0 to 3+. Among nine vaccinates tested, only one had positive staining in the left inguinal lymph node with a score of 2+ and three had scores of 1+, 2+ and 3+, respectively, in gastric lymph node. This finding suggests that there is no difference between controls and vaccinates in terms of proportion of PCV2 antigen positive pigs i.e., 1/8 and 1/9 of controls and vaccinates, respectively, positive in the left inguinal lymph node while 2/8 and 3/9 of controls and vaccinates, respectively, positive in the gastric lymph node. However, two of the vaccinates (#6 and 22) had higher intensity of staining in both left inguinal and gastric lymph nodes compared to that of controls.

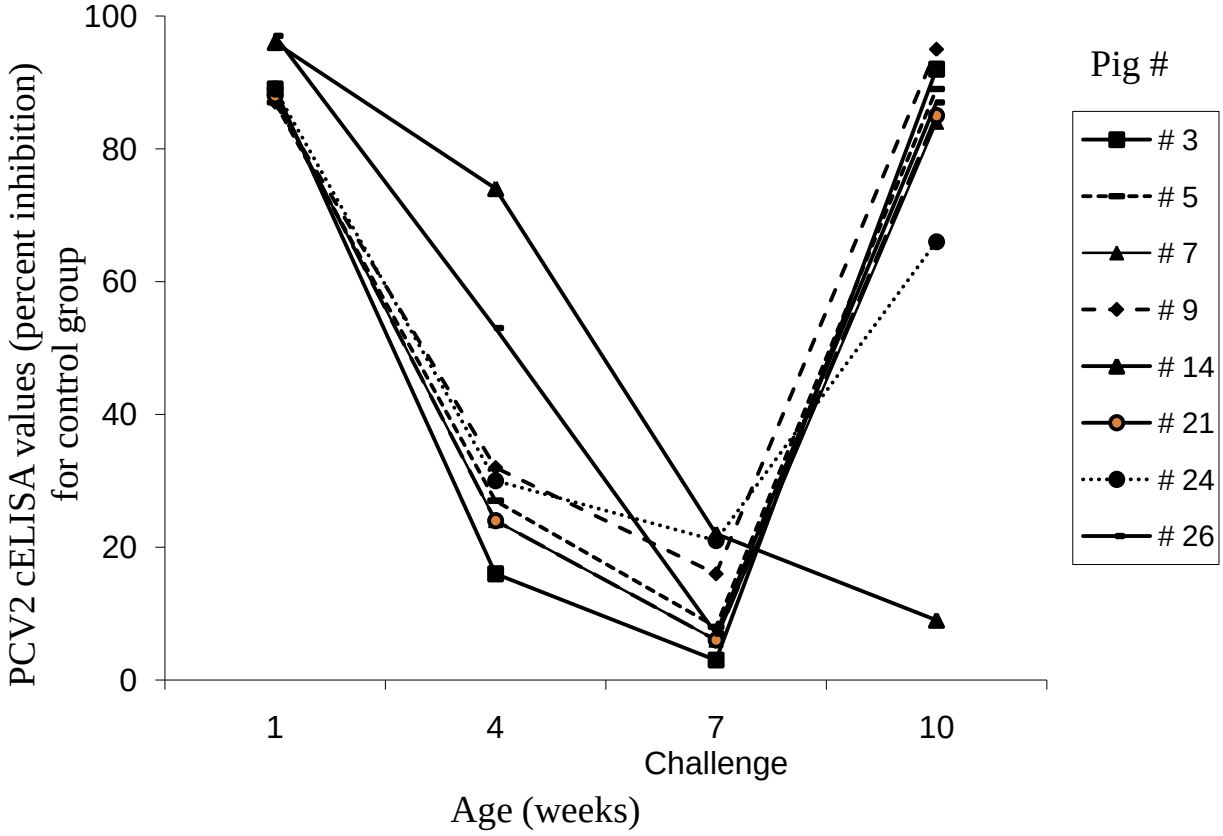

Fig. 2.1 cELISA measuring anti-PCV2 immune responses in individual control pigs receiving PCV2- uninfected PK 15 cell lysate mixed 1:1 with an adjuvant, twice at 3-week intervals, followed by oral challenge with PCV2 inoculums.

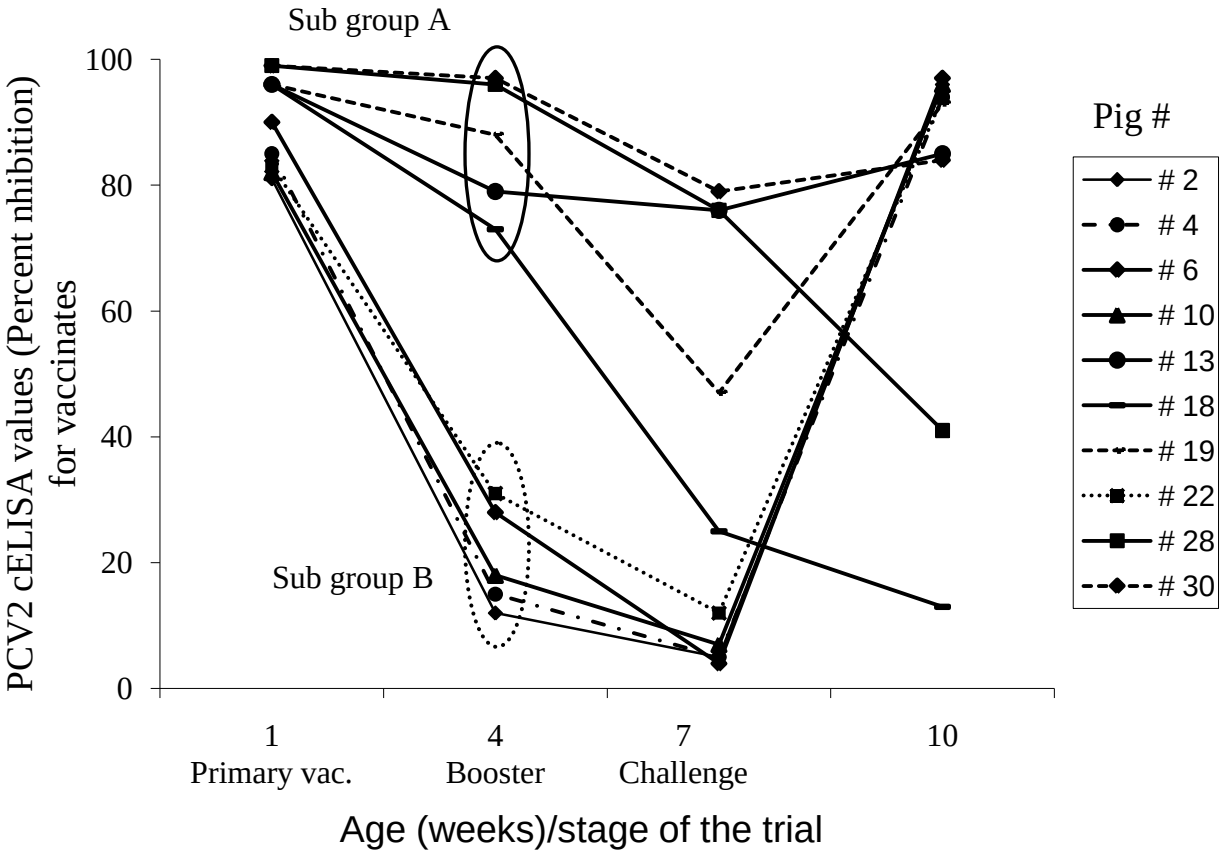

Fig. 2.2 cELISA measuring anti-PCV2 immune responses in individual vaccinates receiving inactivated PCV2- infected PK 15 cell lysate mixed 1:1 with an adjuvant, twice at 3-week intervals, followed by oral challenge with PCV2 inoculums.

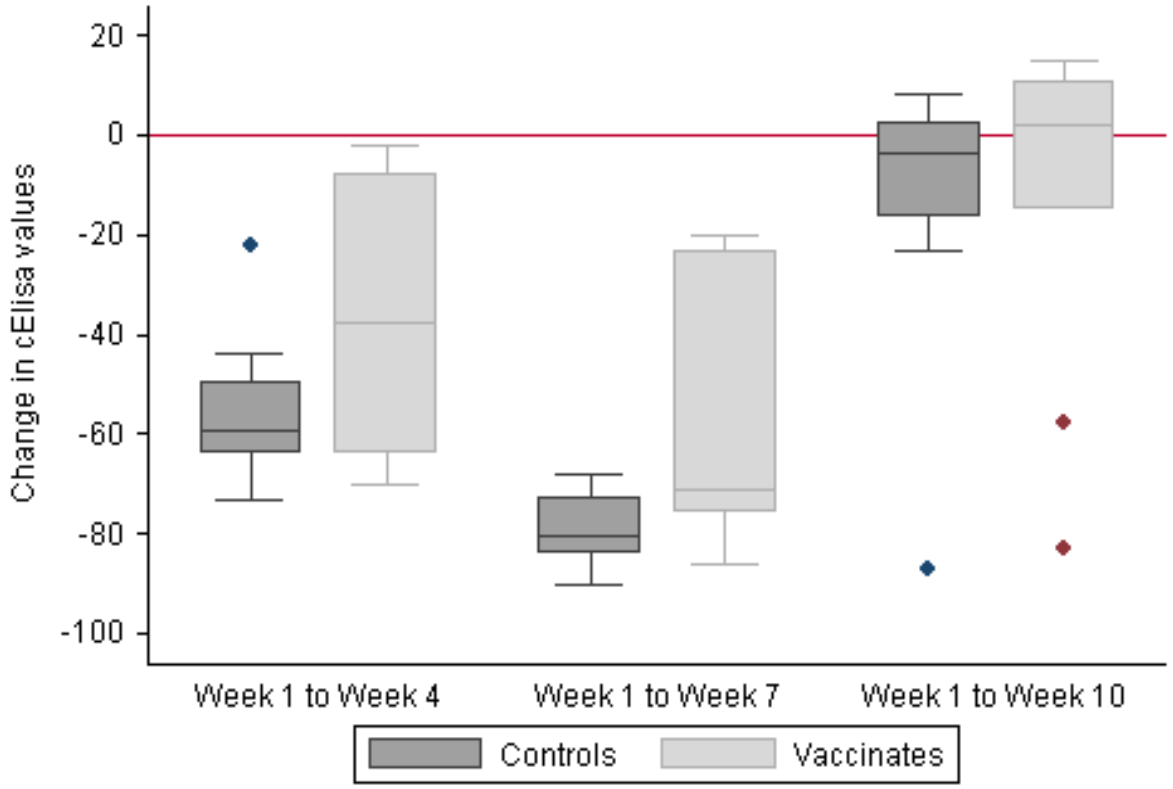

Fig. 2.3 Box and whisker plot for net change in cELISA values in controls and vaccinates following primary vaccination (Week 1 to Week 4), booster (Week 1 to Week 7) and the challenge (Week 1 to Week 10). There was a significantly ($P < 0.05$) smaller decline in cELISA PCV2 antibody levels in vaccinates compared to controls following booster vaccination (Week 1 to Week 7).

Table 2.1

Number of pigs with histological lesions in H & E stained sections of left inguinal and gastric lymph nodes.

Hitologic lesion	Controls (8)[a]	Vaccinates (9)[a]
Lymphoid hyperplasia	8	9
Histiocyte hyperplasia	8	9
Germinal centres	8	9
Lymphoid depletion	0	0
Giant cells	0	0

[a] Number of pigs assayed in each group.

Table 2.2

Immunohistochemistry scores for the presence of PCV2 antigens in two lymph nodes.

Pig ID	Group	Left inguinal	Gastric
3	Control	0	0
5	Control	0	0
7	Control	0	0
9	Control	0	1+
14	Control	0	0
21	Control	0	0
24	Control	0	0
26	Control	1+	1+
2	Vaccinated	0	0
4	Vaccinated	0	0
6	Vaccinated	0	2+
10	Vaccinated	0	0
13	Vaccinated	0	0
18	Vaccinated	0	0
19	Vaccinated	0	0
22	Vaccinated	2+	3+
30	Vaccinated	0	1+

0=No staining, 1+=Minimal, 2+=Moderate, 3+=Extensive

4. Discussion

As expected, given the endemicity of PCV2 infection in commercial swine herds [83, 97], both controls and vaccinated piglets had equally high levels of MDAs for PCV2 prior to vaccination. Although individual animal's responses to vaccination can vary [253], the reason for the two patterns of PCV2 antibody levels among the vaccinated group, i.e., sub groups A and B, following primary vaccination is not clear. To evaluate the immune response to vaccination in the pigs with maternally-derived antibodies, we compared the net change in cELISA values for vaccinates and controls at week 7, following the booster vaccination. The decline in cELISA values in vaccinates was significantly less compared to controls indicating likely a positive effect of immunization.

The aim of this study was to investigate the efficacy of a parenterally administered PCV2 vaccine in the presence of MDAs for PCV2. Possible reasons for the absence of remarkable effect of vaccination on PCV2 antibody titers in the vaccinated piglets include the inhibitory effect of high concentrations of MDAs and/or inadequate amount of vaccine antigen in the final preparation. Mechanisms by which high levels of MDAs can influence the efficacy of live attenuated vaccines include neutralization of the vaccine antigens and formation of MDAs:vaccine antigen complexes that could mask vaccine antigen specific epitopes, inhibition of B cell activation, or enhanced elimination by phagocyte cells [251, 254, 255]. Since an inactivated vaccine was tested, formation of MDAs:antigen complexes is a more likely possibility for the retarded vaccine effect observed in this study [256]. MDAs are known to interfere with humoral response while T cell priming may remain unaffected [251], although cell mediated immune responses following the challenge were not examined in the current study to provide evidence to this effect. A key determinant of MDAs interference is the ratio of vaccine antigen: MDAs, therefore, increasing the vaccine antigen dose may circumvent this issue [255].

Both control and vaccinates, including those pigs with high anti-PCV2 antibody titers (pig # 13 and 30), responded to the challenge inocula that contained the larger amount of PCV2 antigen. This observation demonstrated firstly, that the challenge dose was likely effective and secondly, that the inhibition of immune induction by pre-existing antibodies is reversible by modifying the antigen: antibody ratio, suggesting that improved immune responses may be achieved with increased antigenic mass in the vaccine. Piglets immunized at 4 and 7 weeks of

age with a recombinant pseudorabies virus expressing PCV2-Cap at a dose of 1 x 10^5 $TCID_{50}$ of PCV2 antigens per pig [257], compared to a dose of 4.75 x 10^4 $TCID_{50}$ per pig employed here in 1-week-old piglets, reported a significant anti-PCV2 immune response. Another study examining the influence of MDAs on the efficacy of a PCV2 vaccine using 4-week-old piglets documented a significant reduction in microscopic lesions in lymphoid tissues and viremia in vaccinated pigs [148]. Together, these variable results suggest that the amount of MDAs as well as differences in vaccine formulation, including differences in adjuvants may determine the success of priming for PCV2-specific immunity in piglets with passive immunity. PCV2 isolates with different genetic makeup had shown cross protection against each other [60] and, therefore, the vaccine strain (data not shown) used in the present study could not have influenced the findings.

We conducted a separate experiment (data not shown) with another group of pigs that were vaccinated against PCV2, *Campylobactor coli* and *Helicobactor cerdo*. Therefore, the latter two organisms were included in the challenge inocula in order to challenge the three groups i.e., controls, PCV2 only and PCV2 plus *Campylobactor coli* and *Helicobactor cerdo* vaccinates, simultaneously. Typical clinical signs or gross lesions of PMWS were not observed in either control or vaccinated pigs. Microscopic lesions reported in subclinical PCV2 infection [125] were observed in both controls and PCV2 vaccinates. Further, the two groups were not different on IHC scoring. These observations indicate that while the challenge inoculum likely rendered its effect inducing microscopic lesions there was no progression to clinical PMWS. The vaccine had little or no effect in either inducing a humoral response or preventing histological lesions in vaccinates. A similar study evaluating the efficacy of a PCV2 vaccine by using a different challenge model i.e., intranasally administered dose of 2 x $10^{4.2}$ per pig, reported mild lesions with no significant differences between vaccinated and control pigs [60]. In the present study, a relatively larger challenge dose and multiple days of challenge were employed; there was an initial loading dose of 1.6 x 10^5 $TCID_{50}$ per pig followed by an oral dose of 3.6 x 10^3 $TCID_{50}$ of PCV2 per pig for next 3 days. Previous PCV2 challenge studies have yielded variable results with regard to the production of disease [42, 45, 46, 258]. In the present study it may be that previous exposure/priming, or a protective concentration of MDAs precluded the development of clinical infection. Nevertheless available studies emphasize the frequent difficulties in modeling PCV2 infection in randomly selected pigs from the commercial swine operations that would be candidates for PCV2 vaccination.

In conclusion, we examined the effects of a prototypical inactivated adjuvanted vaccine in inducing active immunity in young piglets against PCV2 with limited success. Further studies using varying vaccine antigen doses in combination with different adjuvants administered to piglets with defined concentrations of MDAs are required to further address the approach of inducing active immunity in young passively immune pigs.

CHAPTER 3: IMMUNOGENICITY OF LAMBDA PHAGE PARTICLES DISPLAYING PORCINE *CIRCOVIRUS* 2 (PCV2) CAPSID PROTEIN IMMUNODOMINANT REGIONS

1. Introduction

Porcine *Circovirus* 2 (PCV2) are small, non-enveloped, single stranded DNA viruses with a circular genome protected by the capsid [22]. One of the diseases caused by the virus, post-weaning multisystemic wasting syndrome (PMWS), sparked severe outbreaks in recent years in Canada causing considerable economic losses to the swine industry [245]. It affects, typically, young pigs between 8-16 weeks of age that are in early grower and late nursery stages with high fatality rates [114]. Virtually all the commercial pig farms are infected [114]. PCV2 viruses are hardy, persisting in the farm environment for long periods of time [75]. Therefore, is considered to be the only effective method of controlling disease outbreaks. At present, there are four commercial PCV2 vaccines available in Canada [96]. All are inactivated vaccines and contain either whole PCV2, PCV1 engineered to express PCV2-Cap replacing its own capsid, or PCV2-Cap expressed from a Baculovirus vector [174, 259] and may contain varying amounts of immune irrelevant proteins. Vaccines containing large amounts of unwanted proteins can lead to adverse injection site reactions [260, 261]. Propagation of PCV2 for vaccine production is time consuming, expensive and laborious process [65]. Further, the inactivation process of PCV2 vaccine antigens may involve corrosive and known carcinogenic chemicals such as beta-propiolactone and may alter the protective immune epitopes, thereby compromising an effective immune response [260]. Hence, there is a need for attempting novel strategies to develop economical, feasible and effective PCV2 vaccines.

Inexpensive vaccines have been developed using filamentous [228, 229, 239, 262] and lytic T4 [230, 231] bacteriophage display systems. They were shown to be immunogenic and potent when administered to laboratory animals. Major coat protein (pVlll) and minor coat protein (plll) were used as fusion partners in developing filamentous phage display vaccines. The T4 display system fuses peptides to the small outer capsid protein (SOC) or the highly immunogenic outer capsid protein (HOC), or both. Lambda phage was suggested as a display vehicle for developing vaccines [235]. The lambda display technique has been used to fuse a wide variety of foreign peptides to bacteriophage surface molecules mainly to target high affinity

tissue ligands [188, 197, 234, 237]. Peptides can be fused to either lambda major tail protein (V) [236], or the head protein D [232]. The latter exists as about 140 trimers bound to the underlying major head protein E. Protein D is considered an ideal fusion partner, because of its surface location and high number of copies available for fusing foreign peptides [188].

In general, phage vaccines offer many attractive features compared to other types of vaccines. They do not replicate in eukaryotic cells and are shown to be safe by the oral route in human volunteers [187]. Large-scale production is inexpensive, simple and extremely rapid [210]. Being particulate antigens, phage is taken up by antigen-presenting cells (APCs) [213] and processed through both MHC I and II pathways [215] ensuring enhanced and balanced immune response. Phage lambda contains many thousands of CpG motifs per genome that would preferably contribute for induction of CMI [263]. Additionally, phage vaccines don't require an adjuvant, can be administered orally or nasally, and are stable under normal storage conditions and a wide pH range (3-11) [216]. The latter characteristics are ideal for a veterinary vaccine. In this study, we test the immunogenicity of lytic bacteriophage lambda displaying four immunodominant regions of PCV2-Cap in an attempt to develop a potential PCV2 vaccine candidate using a novel approach.

2. Materials and methods

2.1.Designing a gene fusion expressing immunogenic regions of PCV2-Cap protein

A particle preparation process incorporating safety and security features was developed for making LDP (lambda display particle), LDNAP (lambda DNA particle) and L2DP (lambda display-DNA particle) with utility as surface display agents and vaccines [31]. Several regions of the PCV2-Cap were previously found to discriminate between common PCV1 and type-specific PCV2 antigens and to represent immunorelevant epitopes for virus type discrimination [32]. We prepared a LDP displaying four regions of the ORF2 capsid (CAP) gene from PCV2. The amino acid sequences for the capsid proteins of ten PCV2 isolates were compared and a consensus sequence was chosen for the isolate with the most common amino acid sequence (GeneBank # DQ629119). The nucleotide sequence corresponding to four regions of this isolate, amino acids 65-87, 113-146, 158-183 and 194-207, were codon optimized for expression in *E. coli* (changing 66 of 97 codons as compared to sequence AF055392), joined by 3 amino acid spacers, and fused to the COOH-end of codon optimized (55 of 110 codons) lambda D gene via a 5 amino acid

linker. The D-CAP gene fusion was made synthetically, confirmed by sequence analysis, and used to make several plasmids (termed pD-CAP), in which the D-CAP gene fusion was either constitutively expressed, or regulated by a temperature sensitive repressor.

2.2. Determining if pigs contain pre-existing anti-lambda antibodies

2.2.1. Raising anti-lambda antisera in pigs

A double CsCl banded unmodified lambda phage was dialysed for 3 hrs each in 1000X volumes of phage dialysis buffer (PDB, 0.3 M NaCl, 0.01 M Tris and 0.05 M $MgCl_2$, pH 7.8) and then phosphate buffered saline (PBS, 0.0036 M KCl, 0.0014 M KH_2PO_4, 0.136 M NaCl, 0.004 M $Na_{2\ hr}PO_4$, pH 7.4). Three piglets 5-6 weeks of age were purchased from a commercial farm, identified individually and maintained at the animal care facility at the Western College of Veterinary Medicine (WCVM). Doses of either 3×10^8, 3×10^9 or 3×10^{10} phage particles per Kg body weight (b.wt.) in 0.5 ml volume were administered three times intradermally to the piglets at three week intervals using an injector gun (Biojector 2000, Bioject Inc. USA). Sera were collected prior to each inoculation and two weeks after the last inoculation. The injection site reactions, rectal temperatures (twice per day for 3 days) following each inoculation, and the general health of the pigs were monitored throughout the experiment. The objective of the use of the three doses employed in this experiment was to gather dose response data to establish the dose for the phage display vaccine.

2.2.2. Gnotobiotic and farm pig sera (field sera)

Sera from eight gnotobiotic pigs provided by Dr. Steve Krakowoka, the Ohio State University, Columbus, Ohio, were used as negative control sera. Sera from fifty-five individual pigs from five farms near Saskatoon, Canada were used as test samples.

2.2.3. Indirect ELISA (iELISA) measuring anti-lambda antibodies

A double CsCl banded lambda phage preparation was dialysed for 3 hr each in 1000X volume of PDB followed by ELISA coating buffer (Na carbonate 0.05M, pH 9.2). Alternative columns (1, 3, 5 etc.) of ELISA plates were coated with 8×10^9 phage particles per 100 µL per well in coating buffer. The remaining columns (2, 4, 6, etc.) were dispensed with 100 µL per well coating buffer. Plates were incubated overnight at 4^oC. Coating buffer was then removed

and plates were washed (5X) with PBS + 0.05% $Tween_{20}$ (PBST). Blocking buffer, PBST + 5% skimmed milk was added in 200 µL volumes per well and plates were incubated for 30 min at 37^{o}C. Serum samples diluted at 1:100 in blocking buffer were added in 100 µL quantities into antigen coated and non-coated wells in duplicate (four wells per sample), plates were maintained overnight at 4^{o}C and then washed (5X) with PBST. Each well was dispensed with 100µL per well of 1:5000 diluted HRP-conjugated protein A (Invitrogen, Carisbad, CA, USA) in blocking buffer and plates were incubated 1 hr at 37^{o}C. After a similar washing step, 100µL per well of 2,2'-Azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt (ABTS) peroxidase substrate (KPL inc, Gaithersburg, Maryland, USA) was added and incubated for 10-20 min at ambient temperature until color developed. The reaction was stopped by adding 50µL of 5% SDS per well. The optical density of the solution in the wells was read at 405 nm. The OD value for each sample was corrected by subtracting the average OD value for two non-antigen coated wells from the corresponding value for two antigen coated wells.

2.3. Demonstrating expression of D-CAP in transformed *E. coli* cells

2.3.1. Protein extraction

E. coli strain R594 F *lac*-3350 *galK 2 galT22 rpsL* 179 IN (*rrnD-rrnE*) l [264] was transformed with pD-CAP. Culture of transformed and untransformed cells were grown overnight at 30^{o}C, diluted 1:20 in fresh media, then grown at 30^{o}C to an OD_{575} = 0.45 and cells were harvested by centrifugation (Beckman, J2-21) at 6000 rpm for 10 min. The cell pellets were resuspended in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl and 10mM Imidazole) containing proteinase inhibitor cocktail (Roche Diagnostics Canada, Laval, Quebec), sonicated (six, 10 s, pulsed bursts, 50% duty cycle) and the cellular debris was removed by centrifugation at 15,000 g for 10 min. Samples were immediately frozen in liquid nitrogen and stored at -70^{o}C. Protein concentrations were estimated by Bradford method [252].

2.3.2. Dot blot assay

Diluted protein extracts from transformed, and untransformed *E. coli* cells, partially purified PCV2 antigen from pig kidney (PK-15) cell line infected with PCV2, and a lambda phage preparation in 1 ml quantities were applied and permitted to pass through a Dot-blot apparatus (Bethesda Research Laboratories Inc., Bethesda, MD, USA) with a nitrocellulose

membrane (Schleicher & Schuell BioScience Inc., New Hampshire, USA) pre-soaked in tris-buffered saline (TBS, 20 mM Tris, pH 7.5, 0.5M NaCl). The membrane was removed and blocked in TBS + 0.1% $Tween_{20}$ + 5% Skim milk overnight at ambient temperature. All protein extracts, antibodies and the reagents were diluted in TBS containing 0.04% $Tween_{20}$ (TBST). The membrane was washed (3X) with TBST prior to and between each step of the staining procedure. The washed membrane was incubated for 1 hr at ambient temperature with anti-PCV2 polyclonal antiserum from a gnotobiotic pig diluted at 1:200 followed by biotinylated goat anti-swine serum (Vector Laboratories Inc., Burlingame, CA, USA) diluted at 1:750 and then incubated with streptavidin-alkaline phosphatase (Bio-Rad Laboratories, Hercules, CA, USA) diluted at 1:1500. The membrane was washed (3X) in double distilled water (DW) for 5 min, 10 ml of freshly prepared BCIP/NBT reagent (Invitrogen, Carisbad, CA, USA) was applied as per the manufacturer recommendations, and the membrane was incubated until color developed. The membrane was washed with DW to stop the reaction.

2.3.3. iELISA

Protein extracts from transformed and untransformed *E. coli* cells were serially diluted in coating buffer. The starting dilution (1:5) was adjusted to contain 21.5 µg total protein per 100µl volume. Duplicate wells of an ELISA plate were coated with 100µL of each dilution from the respective protein extracts and incubated overnight at 4^{o}C. Coating buffer was then removed and plates were washed (5X) with PBST. Blocking buffer was added at 200 µL per well and plates were incubated for 30 min at 37^{o}C. Blocking buffer was removed and the plate was washed (5X) with PBST. Anti-PCV2 polyclonal antiserum from a gnotobiotic pig was dispensed at a dilution of 1:100 in PBST, 100 µl per well, and incubated at 37^{o}C for 1 hr. After washing (5X) in PBST, biotinylated protein A (Invitrogen, Carisbad, CA, USA) was added in volumes of 100 µl per well at a dilution of 1:5000 in PBST and the plate was incubated at 37^{o}C for 1 hr. The plate was washed (5X) in PBST and 100 µl per well streptavidin-peroxidase conjugate (ABC reagent, Vector Laboratories Inc., Burlingame, CA, USA) diluted in PBST according to the manufacturer's recommendation was added and the plate was incubated at 37^{o}C for 30 min. 3.3', 5.5'-tetramethylbenzidine (TMB) substrate (KPL Inc., Gaithersburg, Maryland, USA) was added 100 µl per well, after washing the plate (5X) with PBST. The reaction was stopped by adding 50

µl per well of 1M H_2SO_4 after 15 min of incubation at ambient temperature. The optical density of the solution in the wells of the plate was read at 450 nm.

2.4. Preparation of LDP D-CAP vaccine

LB medium (0.5% w/v NaCl, 0.5% w/v Bacto yeast extract, 1% w/v Bacto tryptone), containing 0.01M $MgCl_2$ and 0.01M Tris, pH 7.6, was inoculated with an overnight culture of 594 [pD-CAP] and incubated at 39^{o}C. At OD_{575}=0.1, the culture was infected at a multiplicity of infection of 0.1 with λ*imm*434*cI*. Incubation was stopped at ~3 hpi which was accompanied by sharp drop of OD_{575} and visually observable cell debris. The lysate was then clarified by centrifugation at 10,000 rpm for 10 min in JLA 16.25 rotor in Avanti J-E centrifuge (Beckman Coulter, Inc., Fullerton, CA, USA) and held overnight at 4^{o}C. Viable phage titers of the lysates (2 L volumes in 4 L flasks) were vary in the range of high 10^9 per ml. NaCl and polyethylene glycol (PEG) MW 1600-1750 were added up to 0.5 M and 4% (w/v), respectively, and the lysate was held overnight at 4^{o}C. Phage was pelleted by centrifugation at 8,000 rpm (as above) for 30 min and the pellets were resuspended in buffer (TM, 0.01 M Tris, 0.01 M $MgSO_4$.$7H_2O$ and 0.1 M NaCl, and pH 7.5). The phage suspension was further concentrated by a second PEG pelleting and banded in a 1.5g per ml CsCl gradient run at 45K in SW 60 Ti rotor in L8-M ultracentrifuge (Beckman Coulter, Inc., Fullerton, CA, USA). The phage band was recovered and rebanded.

2.5. iELISA for phage displaying recombinant D-CAP protein

The double CsCl banded phage display vaccine plus an unmodified lambda phage preparation were dialysed for 3 hrs each in 1000X volume of phage dialysis buffer and then ELISA coating buffer. The preparations were serially diluted in coating buffer and the starting dilution was adjusted to contain 3×10^{10} phage particles per 100µL. Duplicate wells of an ELISA plate were coated with 100µL of each dilution from the respective phage preparation and incubated overnight at 4^{o}C, and the ELISA protocol described (2.3.3) was used to detect D-CAP.

2.6. Evaluation of porcine *Circovirus* 2 phage display vaccine

2.6.1. Immunization trial 1

The LDP-D-CAP vaccine and unmodified lambda phage preparations were dialysed for 3 hrs each in 1000X volume of PDB followed by PBS, pH 7.4. Three piglets at 7 week of age were

purchased from a farm where PCV2 vaccination was not practiced, identified individually and maintained at the animal care facility at the WCVM. Two piglets were immunized each with 1×10^{10} vaccine phage particles per Kg b.wt. intradermally three times at 2-week intervals using an injector gun. Total volume was adjusted to 0.5 ml. The remaining pig (control) was injected with the unmodified lambda phage preparation at the same dose regime. Sera were collected prior to each inoculation and 2 weeks after the last immunization. Body weight, general health and the injection site reaction (if any) were monitored throughout the experiment.

2.6.2. Immunization trial 2

The second immunization trial was carried out with twelve piglets at 7 week of age purchased from a farm where PCV2 vaccination is not practiced. Piglets were randomized into vaccinate and control groups based on maternally-derived PCV2 antibody levels to ensure both groups were contained piglets with a similar levels of these antibodies. A similar procedure and dose regimen to trial 1 were used.

2.6.3. Skin testing for delayed type hypersensitivity (DTH) reaction

On the day following the last serum collection in the second immunization trial, both control and vaccinated pigs were injected intradermally on the left ear at two different sites with 250 µg of unmodified lambda phage and partially purified PCV2 antigen from infected PK-15 cells in 0.1 ml PBS using a 28-gauge needle. They were injected similarly on the right ear with 0.1 ml of PBS and 250 µg of uninfected PK-15 cell lysate in 0.1 ml PBS to serve as placebo for the respective antigens. One control and one vaccinated pig received 250 µg of Phytohemagglutinin-P (PHA-P, Sigma-Aldrich Canada Ltd. Oakville, Ontario) in 0.1 ml PBS on the right ear as a positive control. Skin biopsies were collected using 8-mm biopsy punch (Acu-punch, Acuderm inc., USA) 48 hr post-injection, fixed in 10% formol saline, embedded in paraffin, sectioned at 5µm and stained with haematoxylin-eosin (H & E). The stained tissues were evaluated without knowledge of the identity of the sample on a scale of 0 to 3+ by a pathologist. The scale is 0=negative, 1+=minimal, 2+=moderate, and 3+=Extensive.

2.6.4. iELISA measuring anti-lambda and anti-PCV2 antibodies

Anti-lambda antibodies were measured by iELISA as previously described in 2.2.3. Anti-PCV2 antibodies were detected by iELISA as previously described [107]. Briefly, ELISA plates were coated with partially purified PCV2 antigen from PCV2 infected PK-15 cell lysate and uninfected PK-15 cell lysate in alternative columns of wells as positive and negative antigens, respectively, and their optimum concentrations were determined by checkerboard titration. Plates were incubated overnight at 4^{o}C. Coating buffer was then removed and plates were washed (5X) with PBST. Blocking buffer, PBST + 0.2% gelatin (Sigma-Aldrich Inc., St. Louis, MO, USA) was added 200 µL per well and plates were incubated for 30 min at 37^{o}C. Serum samples diluted at 1:100 in PBST were added into wells coated either with positive or negative control antigen, 100 µL per well in duplicates (four wells per sample). Plates were incubated at 37^{o}C for 1 hr and then washed (5X) with PBST. The remaining steps of the ELISA protocol are as described in 2.2.3.

2.6.5. Virus neutralization assay (VNA)

PCV2-specific neutralising antibodies in three pigs i.e., one control and two vaccinated pigs prior to and after the last immunization were measured by VNA as previously described [78, 265]. Briefly, each serum sample was heat inactivated at 56^{0}C for 30 min and serially diluted twofold up to 1:1024 in minimum essential medium (MEM). An equal volume of PCV2 adjusted to contain 300 $TCID_{50}$ per 0.1 ml was added to serum samples and incubated 2 h at 37^{0}C. The mixtures were added to a flat-bottomed 96-well microtiter plate that was seeded with 1.5 X 10^{5} PK-15 cells per well and incubated at 37^{0}C for 6 hr in a 5% CO_2 atmosphere. The plate was then incubated for 48 hr under similar conditions and the resulting monolayers were treated with 300 mM D-glucosamine (Sigma-Aldrich Inc., St. Louis, MO, USA) 25µl per well for 10 min. Cells were washed twice with 100µl of MEM per well and replenished with 200µl of MEM per well and incubated as before for an additional 16 hr. The monolayers were washed once with PBST, allowed to air dry for 1 hr and fixed in 80% cold acetone for 10 min. The air dried cells were stained for PCV2 antigens using immunoperoxidase assay [83]. The neutralization titers were expressed as the reciprocal of the highest serum dilution that completely blocked the infection in PK-15 cells, as compared to virus control.

2.6.6. Immunoblots

The D-CAP phage display vaccine and the unmodified lambda phage preparations were dialysed for 3 hrs in 1000X volume of phage dialysis buffer. The phage preparations and a PCV2 infected cell lysate were adjusted to contain equal amount of total protein and boiled in SDS-PAGE sample buffer (0.0625M Tris pH 6.8, 4.65% (w/v) SDS, 20% (v/v) glycerol, 10% (v/v) β-mercaptoethanol, 0.025% (w/v) bromophenol blue) for 5 min and separated by 12% polyacrylamide gel in Mini-PROTEAN 3 electrophoresis cell (Bio-Rad Laboratories, Hercules, CA, USA) along with protein markers (Bio-Rad Laboratories, Hercules, CA, USA). Proteins were transferred to nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA, USA) at 400 mA for 90 min. The membranes were blocked overnight in Tris-buffered saline (TBS, 20 mM Tris, pH 7.5, 0.5M NaCl) containing 0.1% $Tween_{20}$ and 5% skimmed milk. The blots were washed twice in TBST for 5min followed by reacting either with anti-PCV2 polyclonal from a gnotobiotic pig or with serum taken from a pig prior to and after vaccination, at a dilution of 1:200 in TBST at ambient temperature for 1 hr. After washing the membranes 3X (for 5, 15, and 5 min) with TBST, they were incubated with biotinylated protein A (Invitrogen, Carisbad, CA, USA) at a dilution of 1:1500 in TBST for 1 hr at ambient temperature. The membranes were rinsed 3X (as above) with TBST and incubated with streptavidin-alkaline phosphatase (Bio-Rad Laboratories, Hercules, CA, USA) at a dilution of 1:1500 in TBST for 1.5 h at ambient temperature. The blots were washed 3X in DW for 15 min and the reaction was visualized by applying freshly prepared BCIP/NBT reagent (Invitrogen, Carisbad, CA, USA) as per the manufacturer recommendation. The color development was stopped by rinsing the membranes with DW.

3. Results

3.1. Raising anti-lambda hyperimmune sera in pigs

There was a gradual increase in anti-lambda antibody levels following the second and third immunization among three pigs that received 10-fold varying doses of lambda phage antigen (Fig. 3.1 & 3.2) during the experiment undertaken to raise anti-lambda hyperimmune sera. A dose dependent response was evident at the second immunization. However, the third immunization eliminated the dose dependency.

3.2. Determining if pigs contain pre-existing anti-lambda antibodies

There was no significant difference ($p > 0.05$) between mean anti-lambda antibody levels for 55 individual pig serum samples (field sera) from five different farms and eight gnotobiotic pig sera that were employed as presumptive negative anti-lambda antisera control (Fig 3.3). Field and negative control sera (Fig. 3.3) were significantly different ($p < 0.05$) from the hyperimmune serum sample (positive control); sera from the pig received 3×10^{10} lambda particles per Kg b.wt. following third immunization, see Fig. 3.2).

3.3. Assessing for the expression of D-CAP fusion protein in 594 [pD-CAP] cell extracts

The dot blot assay (Fig 3.4) showed a greater reaction with the 594 [pD-CAP] extract (b) compared to a slight background reaction with *E. coli* 594 extract (a). As expected, PCV2 antigen reacted most intensely with anti-PCV2 polyclonal antiserum from a gnotobiotic pig (c). In contrast, there was no reactivity of lambda phage with the anti-PCV2 polyclonal antiserum (d). Similarly, ELISA (Fig 3.5) showed higher ODs throughout a series of dilutions from 594 [pD-CAP] extract compared to that of *E. coli* 594 extract, most intensely at 1:5 dilution.

3.4. Demonstrating D-CAP displayed in LDP vaccine

The ELISA data showed significantly higher reactivity throughout a series of phage display vaccine dilutions with anti-PCV2 polyclonal antiserum from a gnotobiotic pig compared to corresponding dilutions containing an equal number of unmodified phage lambda particles (Fig. 3.6) indicating the presence of recombinant PCV2-CAP protein in the phage display vaccine particles.

3.5. Anti-lambda and anti-PCV2 antibody response following phage display vaccination

Evaluation of immune responses to lambda phage coat proteins in pigs receiving either LDP-D-CAP or unmodified phage preparations revealed a very low level of anti-lambda antibodies prior to the experiments that increased gradually following each immunization (Fig. 3.7 & 3.9). This finding is in agreement with our previous observation that pigs contain very low-to undetectable anti-lambda antibodies (Fig. 3.3).

Three piglets in the first vaccine trial had low levels of anti-PCV2 antibodies (Fig. 3.8). One vaccinate responded at primary (open bar) and the other responded at the second (closed bar) vaccination. Third immunizations resulted in no increase in the response in one of the vaccinates (open bar) while the other had a diminished anti-PCV2 antibody level (closed bar). The control pig had low and stable levels of PCV2 antibodies throughout the experiment (hatched bar).

Grouping pigs based on the level of anti-PCV2 antibodies prior to vaccination in the second trial ensured that both control and immunized pigs had a similar level of maternally-derived anti-PCV2 antibodies (MDPCV2A) at the beginning of the experiment (Fig. 3.10). The vaccinates had significantly ($p < 0.05$) higher anti-PCV2 immune responses following primary vaccination, which plateaued following the second vaccination and slightly decreased after the third vaccination. The level of anti-PCV2 antibodies among controls remained low and constant throughout the experiment. Two of the LDP-D-CAP immunized pigs tested had considerably higher PCV2 neutralizing antibody titers compared to a pig that received lambda control inoculum (Table 3.1).

3.6. Monitoring body weight, general health and injection site reactions

Both groups increased their body weight by ~17 Kg in 2 week period. There was no injection site reaction to lambda control or display vaccine preparation.

3.7. Immunoblots

The blots reacted with anti-PCV2 polyclonal antiserum from a gnotobiotic pig (Fig. 3.11a) or with antiserum from a pig vaccinated with D-CAP phage particles (Fig. 3.11c) revealing the D-CAP (arrow) and the 233 amino acid PCV2-Cap (triangle) bands. The blot reacted with serum collected prior to vaccination (Fig. 3.11b) shows a lightly stained D-CAP (arrow) without detection of a PCV2-Cap band.

3.8. DTH reaction

The representative DTH reaction scores are shown in Fig. 3.12. All pigs vaccinated with LDP-D-CAP (3 of 3) were hypersensitive in the DTH assay (Table 3.2) to both lambda phage and PCV2 antigens compared to their respective placebo. One of the LDP-D-CAP vaccinated

pigs recorded an intensive reaction (3+) to PCV2 antigen. Within the control group, 3 of 5 pigs were sensitive to lambda while 2 of 4 showed reactivity to PCV2 antigen.

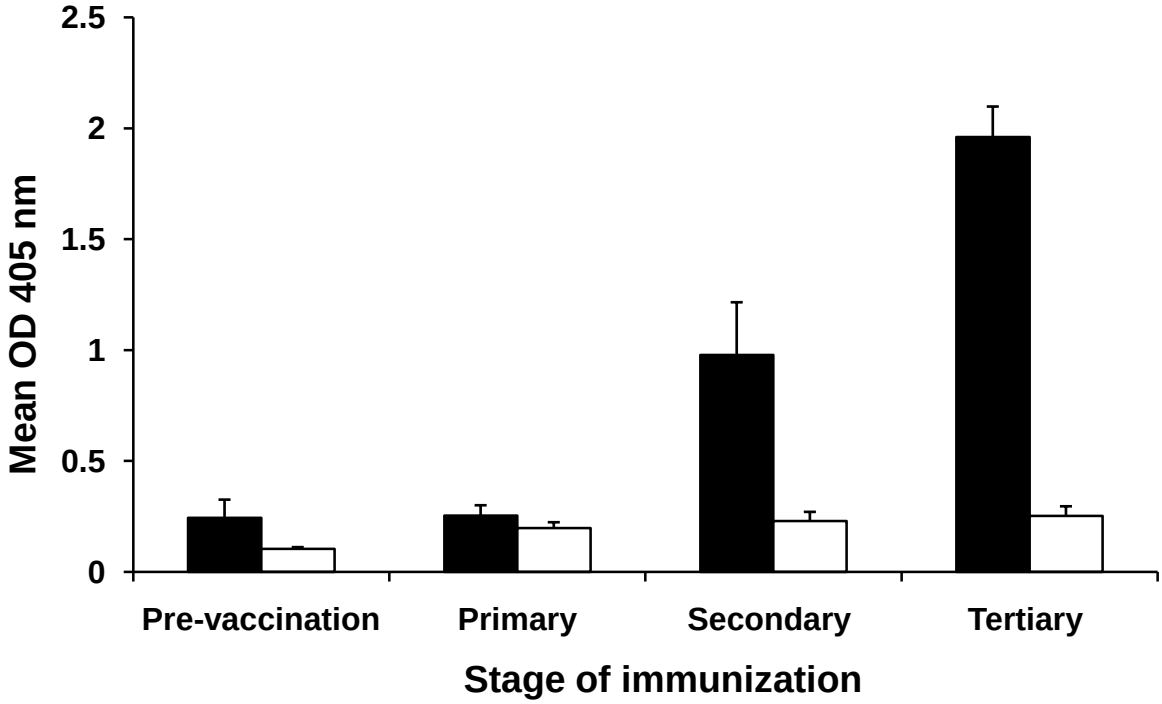

Fig. 3.1 The anti-lambda immune response measured by ELISA in three pigs each of which received 3×10^8, 3×10^9 or 3×10^{10} phage particles per Kg b.wt. (3X), at 2-week intervals in comparison to the background OD (open bars). Values are the mean + standard error (SE).

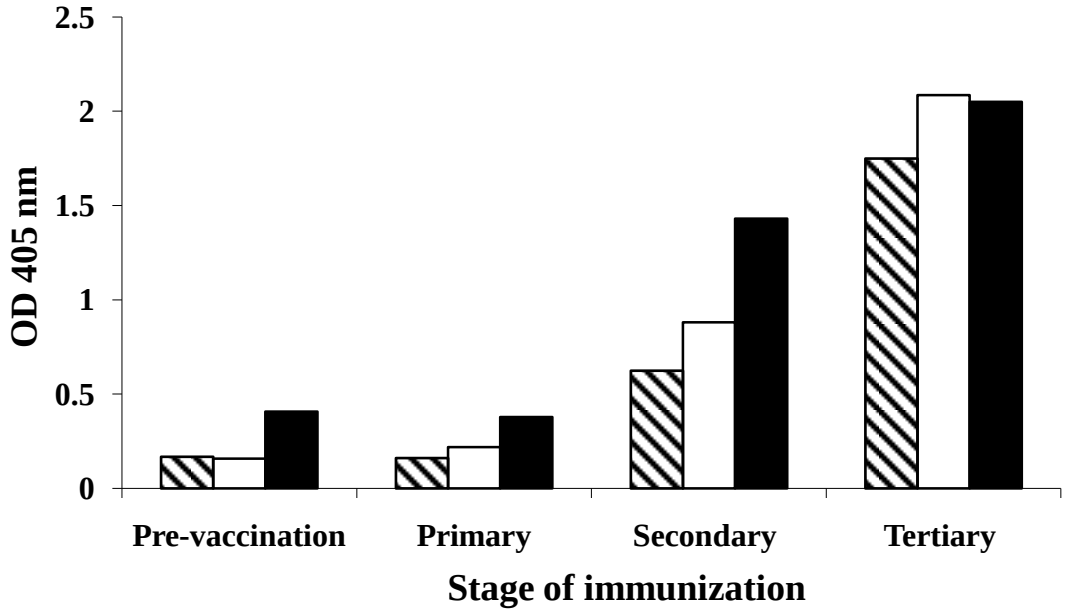

Fig. 3.2 The **a**nti-lambda antibody response measured by ELISA in individual pigs receiving 3×10^{8} (hatched bar), 3×10^{9} (open bar) or 3×10^{10} (closed bar) phage particles per Kg b.wt. (3X), at 2-week intervals.

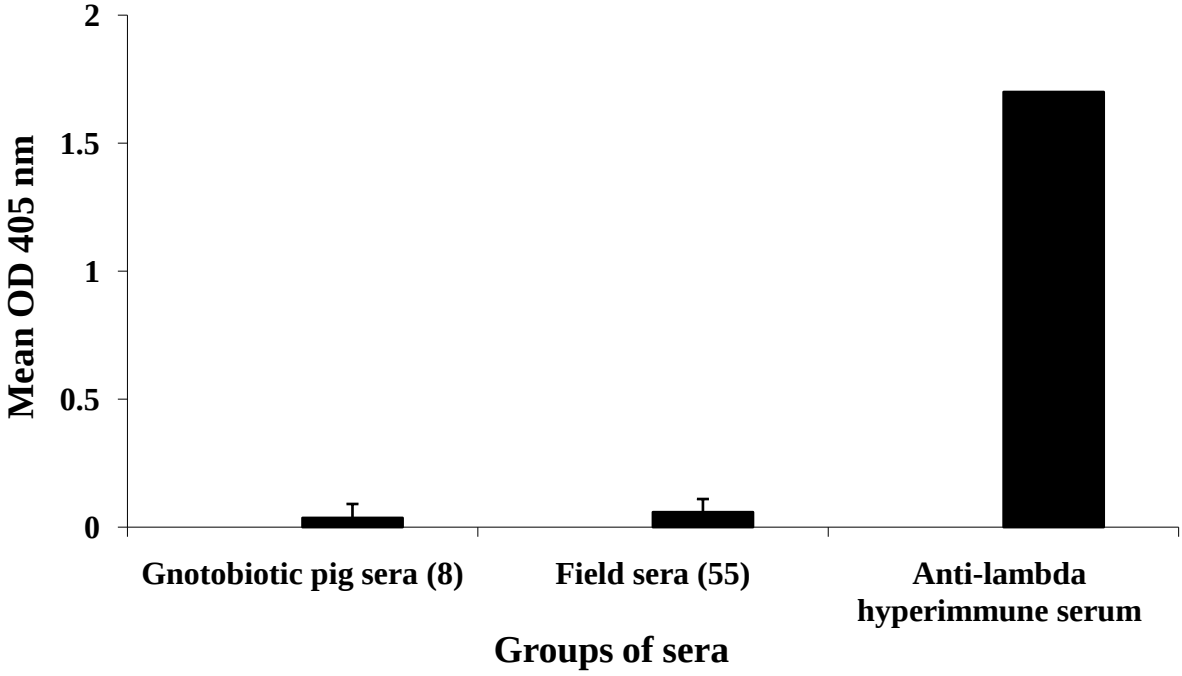

Fig. 3.3 Pre-existing anti-lambda antibody levels in sera from eight gnotobiotic pigs (negative control) and sera from 55 conventional pigs from five different farms (field sera) were measured by ELISA and compared to an anti-lambda hyperimmune serum (positive control). Values are the mean + standard error (SE) for gnotobiotic and field sera.

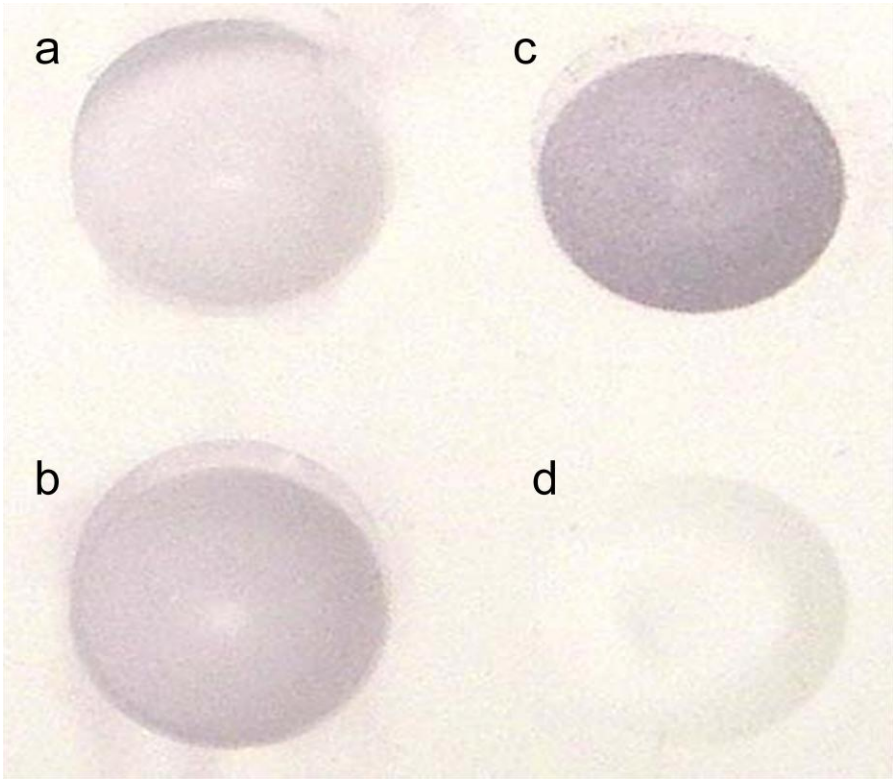

Fig. 3.4 Dot-blot assay detecting D-CAP fusion protein in *E. coli* cell extract using anti-PCV2 polyclonal antiserum from a gnotobiotic pig; a) *E. coli* R594 cell extract, b) *E. coli* 594 [pD-CAP], c) PCV2 antigen-positive control and d) lambda phage antigen-negative control. The total protein applied per blot site was: a) 165 µg b) 165 c) 5 µg of partially purified PCV2 antigen from PK15 infected cells and d) 1×10^{8} unmodified phage particles.

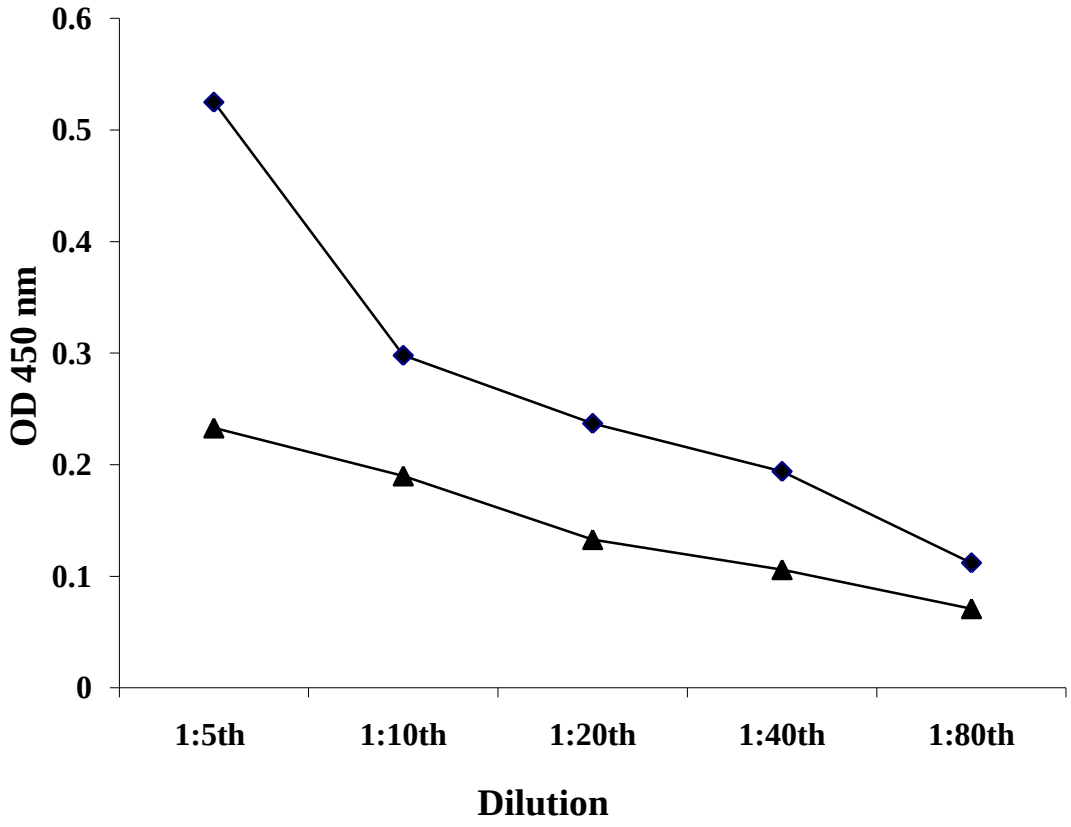

Fig. 3.5 Results of ELISA measuring D-CAP fusion protein in an *E. coli* cell extract using anti-PCV2 polyclonal antiserum from a gnotobiotic pig. Serial dilutions were made of a protein extract obtained from R594 [pD-CAP] *E. coli* cells (■). Corresponding dilutions were made of a protein extract obtained from R594 *E. coli* cells (▲). The total protein coated per well at $1:5^{th}$ dilution was 21.5 µg.

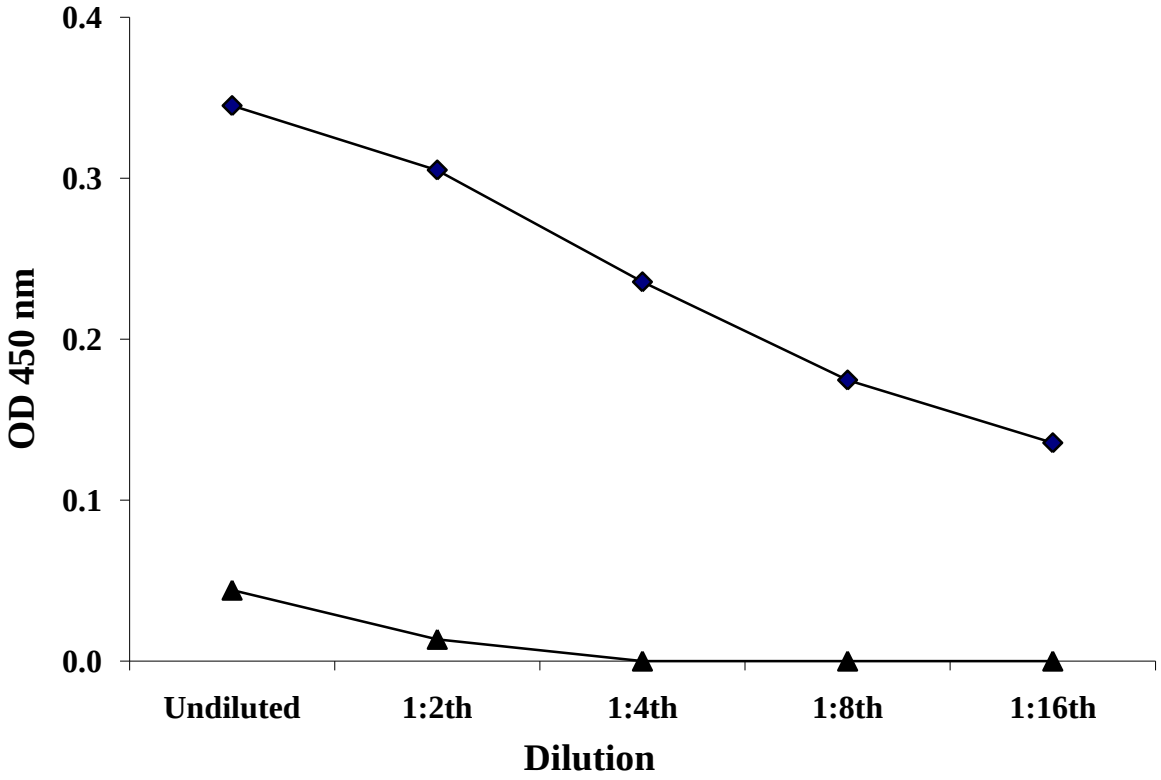

Fig.3. 6 Results of ELISA measuring phage displayed D-CAP fusion protein using anti-PCV2 polyclonal antiserum from a gnotobiotic pig. Serial dilutions of LDP-D-CAP vaccine (■) were made. Corresponding dilutions were made of an equivalent number of unmodified lambda phage particles (▲). The number of phage particles in the undiluted preparation was 3×10^{10} per well.

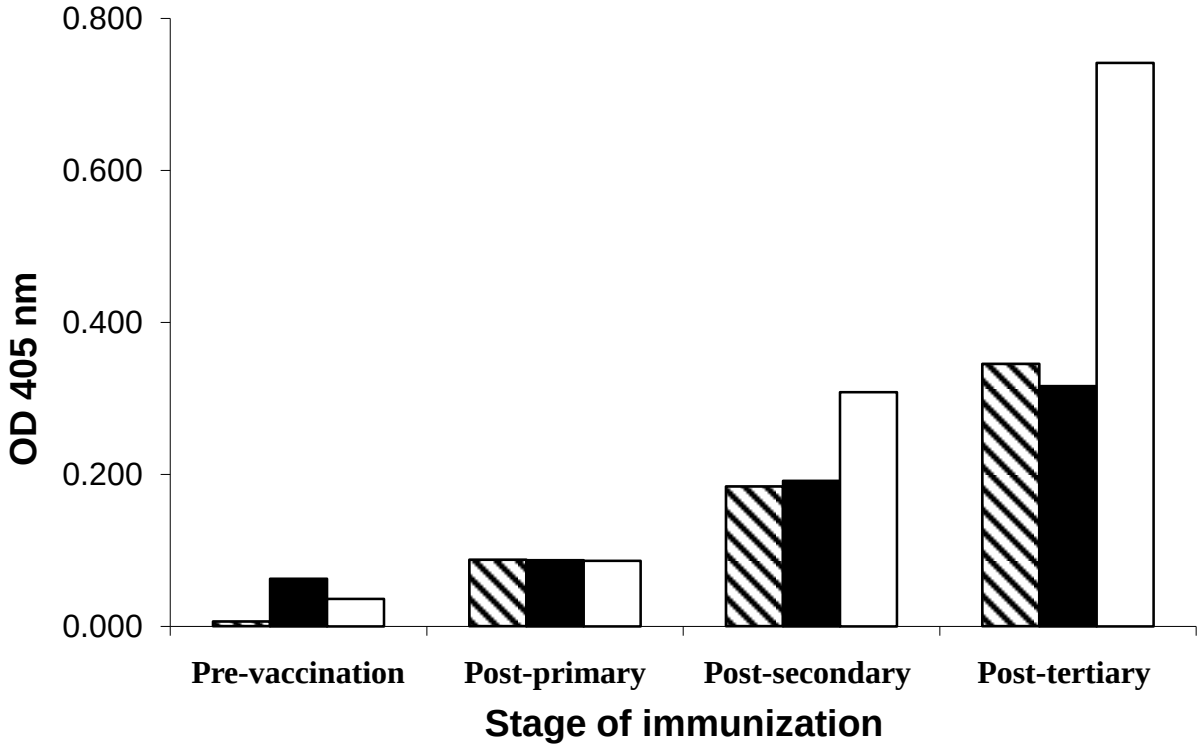

Fig. 3.7 Results of ELISA measuring anti-lambda antibody response in two vaccinated pigs (closed & open bars) receiving 1×10^{10} LDP-D-CAP per Kg b.wt. intradermally without incorporating an adjuvant, 3X at 2-week intervals, and a control pig (hatched bars) receiving similar doses of unmodified lambda particles.

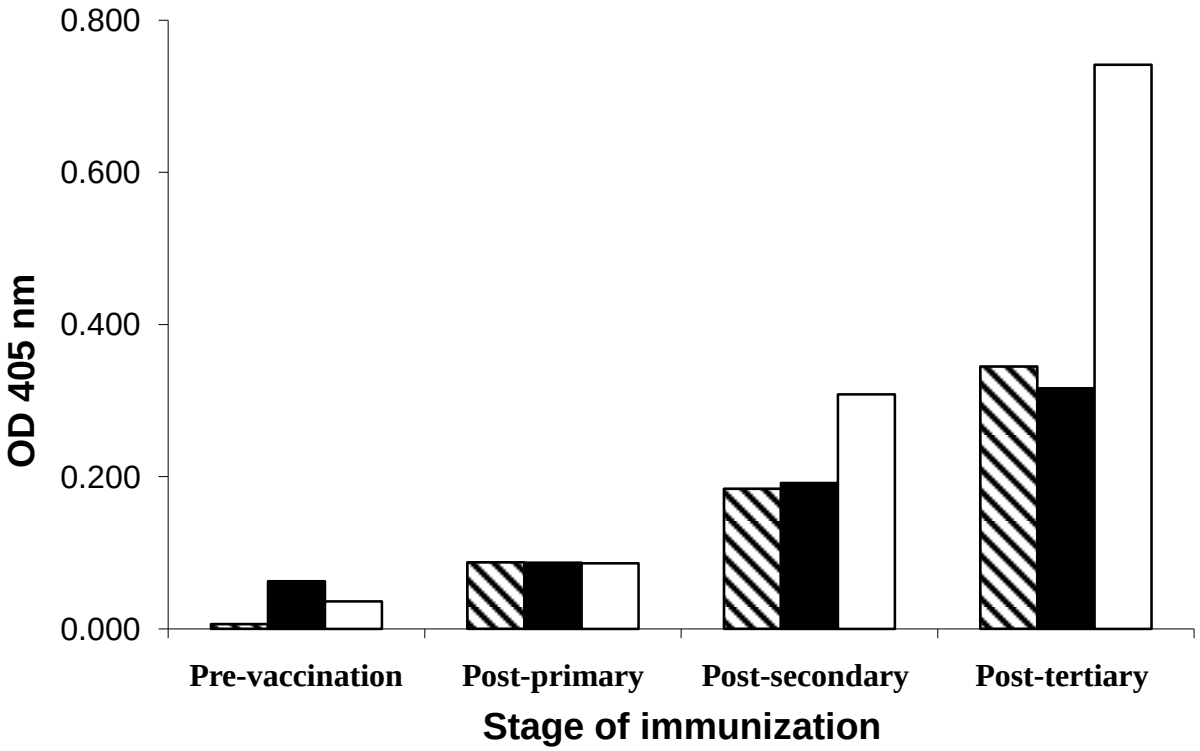

Fig. 3.8 Results of ELISA measuring anti-PCV2 antibody responses in two vaccinated pigs (closed & open bars) receiving 1×10^{10} LDP-D-CAP per Kg b.wt. intradermally without incorporating an adjuvant, 3X at 2-week intervals, and a control pig (hatched bars) receiving similar doses of unmodified lambda particles.

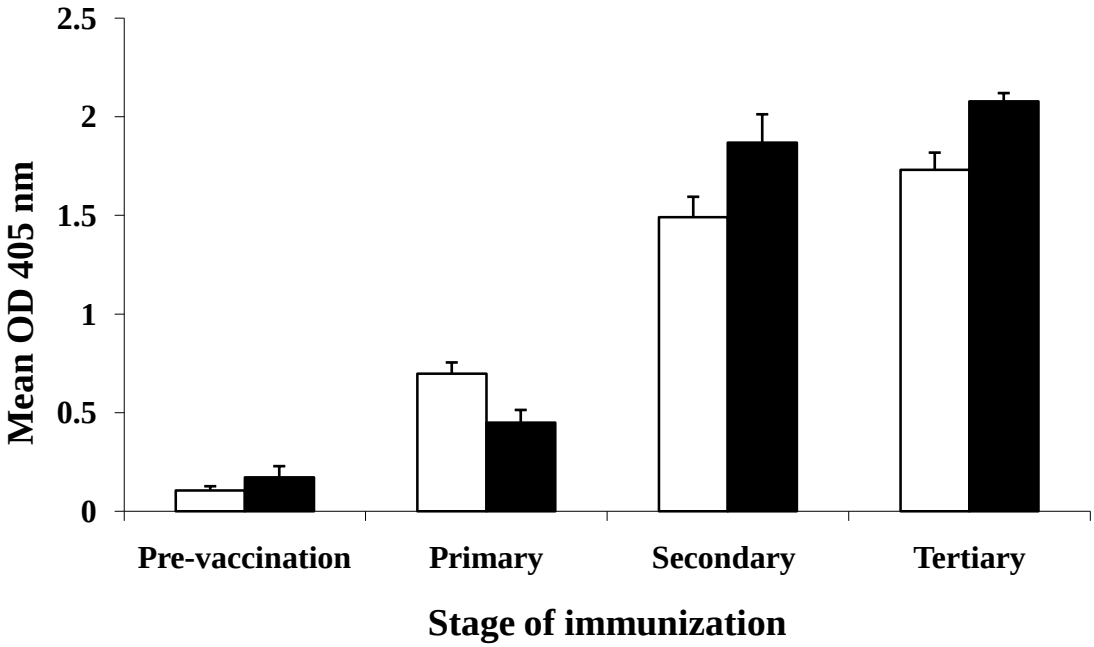

Fig. 3.9 Results of ELISA measuring anti-lambda immune antibody responses in six vaccinated pigs receiving 1×10^{10} LDP-D-CAP per Kg b.wt. intradermally without incorporating an adjuvant, 3X at 2-week intervals (closed bars), and six controls (open bars) receiving similar doses of unmodified lambda particles. Values are the mean + standard error (SE).

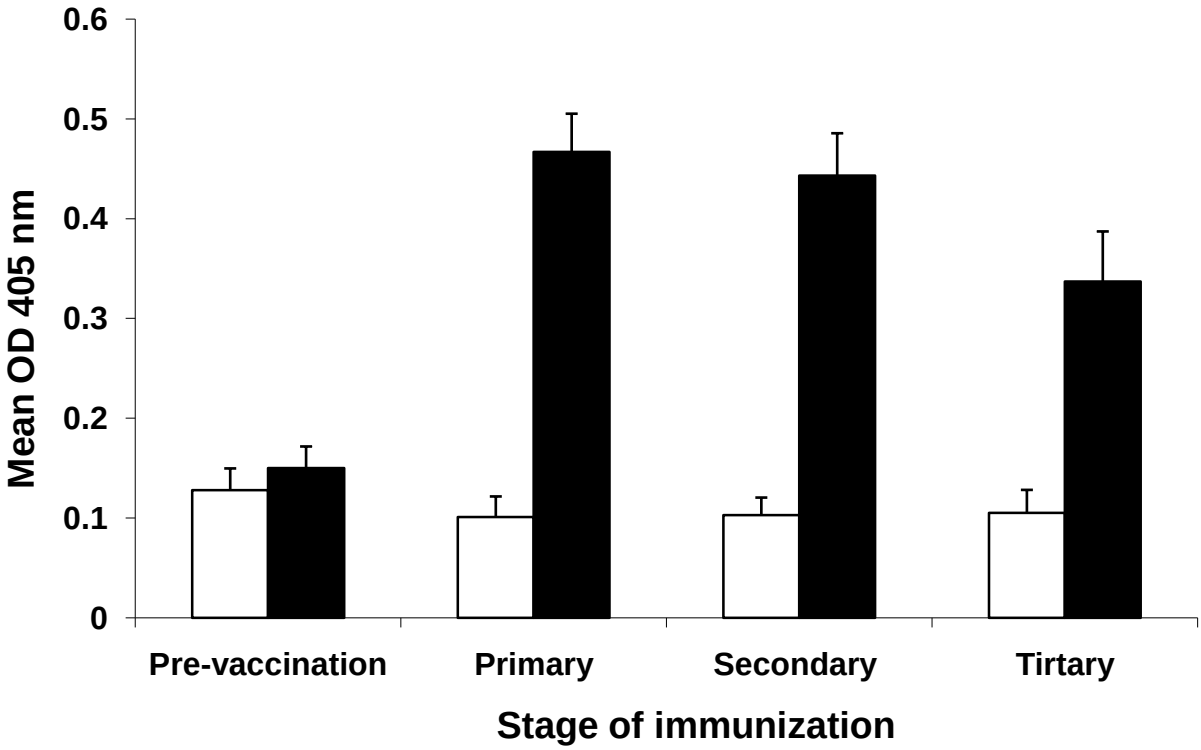

Fig. 3.10 Results of ELISA measuring anti-PCV2 antibody responses in six vaccinated pigs receiving 1×10^{10} LDP-D-CAP per Kg b.wt. intradermally without incorporating an adjuvant, 3X at 2-week intervals (closed bars), and six controls (open bars) receiving similar doses of unmodified lambda particles. Values are the mean + standard error (SE).

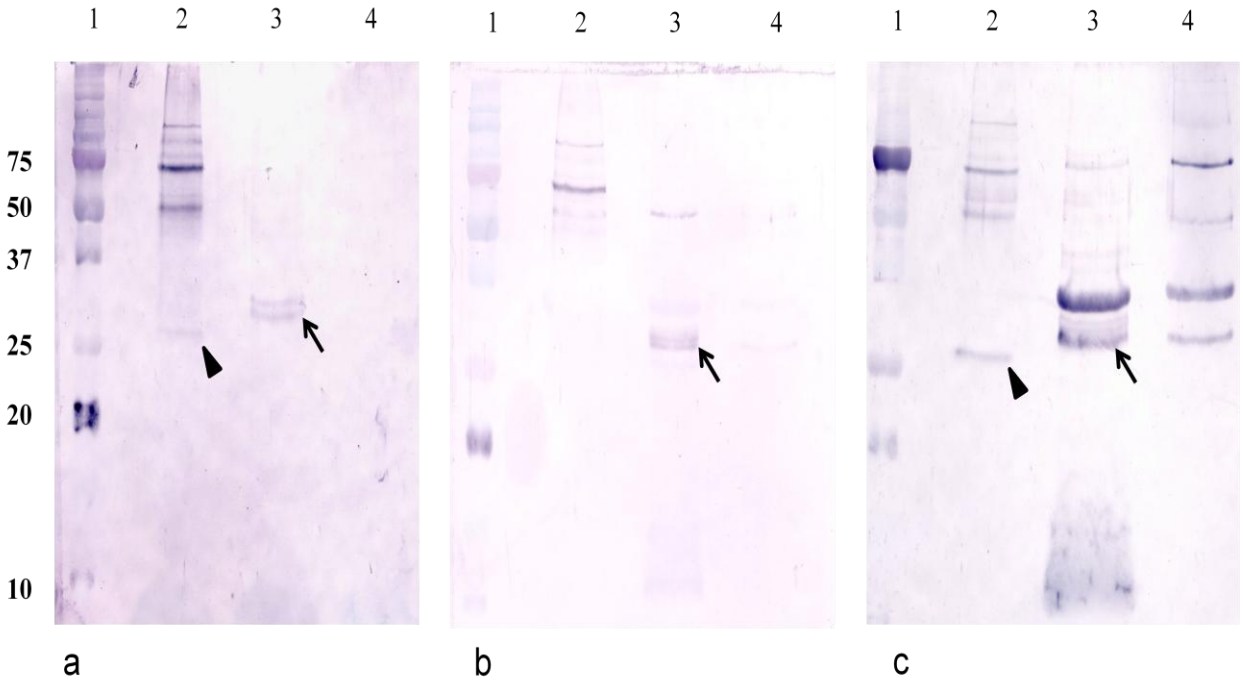

Fig. 3.11 Western blots demonstrating phage displayed D-CAP fusion protein using: (a) anti-PCV2 polyclonal antiserum from a gnotobiotic pig; (b serum from a pig prior to vaccination; and (c) serum from a pig after vaccination. Lanes; 1) Protein mass marker, 2) partially purified PCV2 antigen from PK-15 infected cells, 3) LDP-D-CAP from heat-disrupted phage particles, and 4) similarly disrupted unmodified phage particles. Arrow indicates D-CAP protein. Triangle points to PCV2 cap protein (positive control).

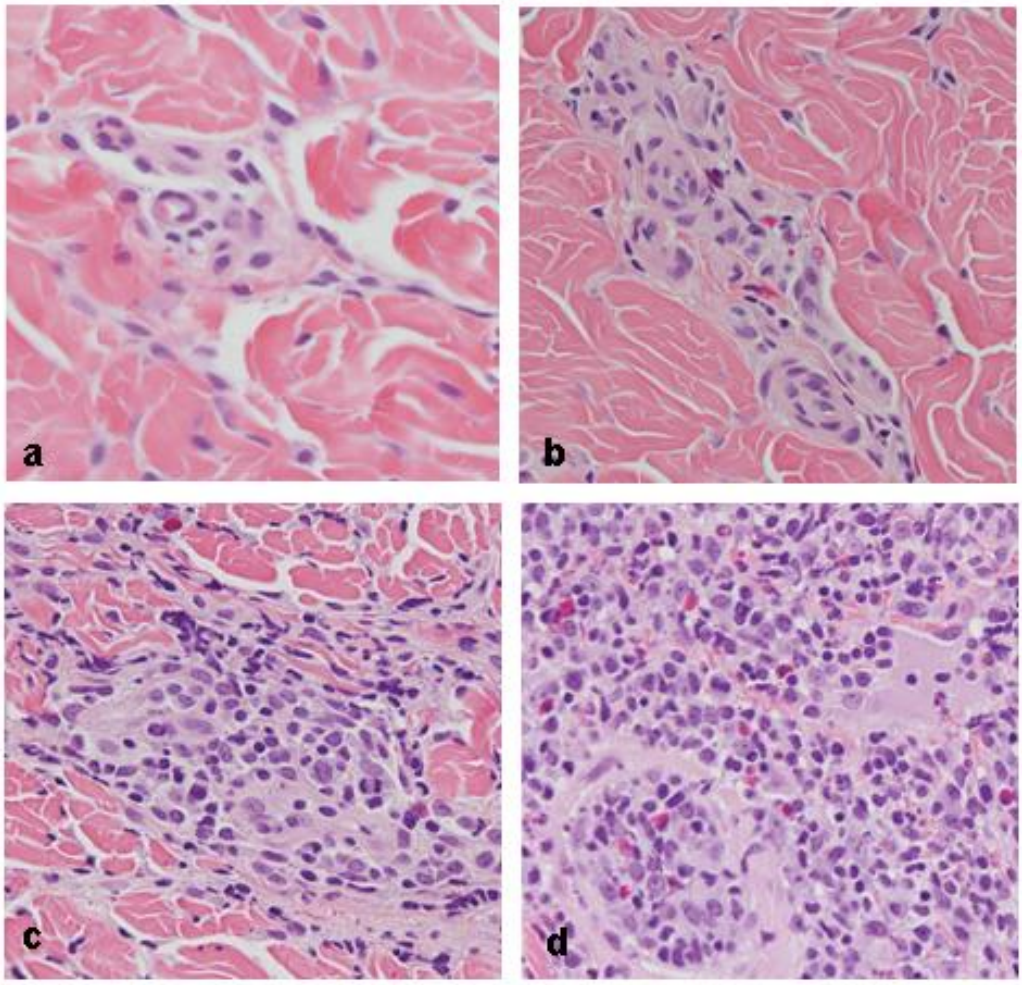

Fig. 3.12. Photographs of representative DTH reaction scores: (a) 0, (b) 1+, (c) 2+ and (d) 3+ based scoring of H & E stained skin biopsy tissues. Original magnification (a-d): Magnification ×50. The scale is 0=negative, 1+=minimal, 2+=moderate, and 3+=Extensive

Table 3.1 PCV2 neutralizing antibody titers of two pigs immunized with LDP-D-CAP compared to one pig receiving lambda control.

Group	Pre-immunization	Post-immunization
LDP-D-CAP	2	256
LDP-D-CAP	2	256
Lambda control	16	2

Table 3.2 Delayed type hypersensitivity (DTH) reaction scores in H & E stained skin biopsy tissues from pigs vaccinated with lambda phage and partially purified PCV2 antigens from PK-15 infected cell lysate and their respective placebo; PBS and uninfected PK-15 lysate following phage display vaccine trial.

Group	Antigen		Placebo	
	Phage	PCV2	PBS	PK15
Vaccinated	+/-	2+	0	(+/-)
Vaccinated	1+	3+	0	(+/-)
Vaccinated	1+	(+/-)	0	0
Control	1+	N/R[a]	0	0
Control	0	0	N/R[a]	N/R[a]
Control	0	1+	0	0
Control	1+	0	0	0
Control	1+	2+	0	0

[a] Not recorded

0=Negative, (+/-)= Slight or no, 1+=Minimal, 2+=Moderate, and 3+=Extensive

Phytohemagglutinin-P (positive control) recorded a score of 3+

4. Discussion

Phage display was examined as a potential vaccination strategy for minimizing the economic losses due to PMWS caused by PCV2 infection. There is renewed interest in developing new biological agents that can replace traditional vaccines [197], [214, 266]. We prepared lambda particles displaying recombinant PCV2-Cap , i.e., LDP-D-CAP. The design of LDP-D-CAP vaccine was based on information showing that immunogenicity of PCV2, relative to PCV1, was mapped to four dominant immunoreactive areas of the PCV2-Cap [14], which were shown to render protection against the disease [259].

We first demonstrated expression of the recombinant D-CAP protein in *E. coli* 594 [pD-CAP] cells by the Dot-blot assay and ELISA. In the Dot-blot assay, PCV2 antigen and the lambda phage served as the positive and negative controls, respectively. In both instances, we used anti-PCV2 polyclonal antisera from gnotobiotic pigs as the primary antibody. Gnotobiotic pigs are born by caesarean section and kept thereafter in a microbe free environment [38, 39]. These pigs were inoculated with PCV2 to raise anti-PCV2 polyclonal antisera and are considered devoid of antibodies against any other microbes. This serum was used throughout the experiments to detect the expression of recombinant D-CAP in *E. coli* cells and on LDP.

Lambda phage possesses an ability to display large D-fusion peptides at high display density [235]. The LDP-D-CAP display phage were concentrated by double CsCl gradient to purify them from lower buoyant density lipopolysaccharides (LPS) and, from contaminating bacterial proteins that are expelled from the CsCl gradient. The reactivity of LDP-D-CAP with anti-PCV2 polyclonal from a gnotobiotic pig by ELISA was significantly higher than that of the unmodified lambda phage preparation indicating that D-CAP fusion proteins were displayed on the LDP-D-CAP vaccine particles.

The background reaction observed in the Dot-blot assay and ELISA with protein extracts from *E. coli* is because of non-specific binding of polyclonal sera with bacterial proteins. We have repeatedly observed a very low intensity reaction between double CsCl purified unmodified lambda phage and anti-PCV2 polyclonal sera from gnotobiotic pigs in our immunological assays. This finding indicates that the CsCl purified lambda phage preparation is free from bacterial contamination.

We tested the LDP-D-CAP in pigs, the end-target species to avoid the swamping effect reported by similar vaccine experiments [240] and the common criticism of having to extrapolate laboratory animal data to the natural host. The approach provided valuable data to establish a dose regimen for intradermal injection in pigs. We addressed an unknown critical issue of whether pigs contain pre-existing anti-lambda antibodies. This information was vital to ensure an effective immune response by phage vaccines without a risk of being suppressed by the pre-existing carrier specific immunity. There are conflicting reports indicating that pre-existing carrier specific antibodies could enhance [267], have no effect [268, 269] or suppress [270] the target vaccine antigen specific immune response.

For testing sera from conventional pigs for anti-lambda antibodies, we first raised anti-lambda hyperimmune sera in pigs as the positive control. This trial was designed in such a way that we would be able to determine the dose response to phage coat antigens and thereby decide the optimum dose of phage display vaccine in the next experiment. Three 10-fold varying doses of lambda phage (3×10^8 [≈0.016 μg phage DNA], 3×10^9, 3×10^{10} per Kg b.wt.) were employed to raise anti-lambda antibodies starting with about 10 Kg piglets.

We employed the ELISA described by March et.al. [240] measuring anti-lambda antibodies in sera collected at different time points during the course of raising hyperimmune sera. There was a gradual and significant increase in anti-lambda antibodies following the second and third immunizations in individual animals. The dose dependent immune response was clearly evident at the second immunization. The third dose eliminated any significant difference in immune response between the lowest and the highest dose suggesting that the lowest dose should be sufficient for vaccination when unmodified lambda phage is administered three times.

For addressing the important question of whether pigs have pre-existing anti-lambda antibodies, we analysed the ELISA data pertaining to field samples (conventional pig sera) along with the presumed negative (gnotobiotic pig sera to PCV2) and the positive control (anti-lambda hyperimmune serum). There was no significant difference ($p > 0.05$) between the negative standard and the test sera. However, both groups were significantly different ($p < 0.05$) from the positive standard. These data indicate pigs contain very low (or no) anti-lambda antibodies. This finding assures that phage vaccines can be administered to pigs without the risk of suppression of the target vaccine antigen specific immune response by pre-existing carrier immunity.

We undertook LDP-D-CAP immunization trial in 7-wk old pigs using an intermediate dose of 1×10^{10} vaccine phage particles per Kg b.wt. in 0.5 ml administered three times at 2 week intervals via an intradermal route using an injector gun to increase the chance for uptake of phage vaccine particles by dendritic cells which possibly enhance an immune response [271]. The injector gun ensured the accurate delivery of small quantities of vaccine. Our dose regime ensures adequate time and number of immune stimulations to study the pattern of vaccine antigen specific immune response (anti-PCV2) against the carrier specific (anti-lambda) response. A similar experiment, in a phage DNA vaccine trial employed doses of 5×10^{9} phage per mouse and 4×10^{10} phage per rabbit [240]. There was a evoke a significant immune response in pigs at 3×10^{8} unmodified lambda, or 1×10^{10} LDP-D-CAP phage per 1 Kg b. wt. pig. Assuming weight of a mouse is 30g, we were able to vaccinate using $1/550^{th}$ or $1/16^{th}$ lower doses of unmodified lambda or LDP-D-CAP phage particles per Kg b.wt., respectively, compared to a dose of 5×10^{9} phage per mouse employed by a lambda DNA vaccine experiment [240]. The pattern of carrier specific (anti-lambda) immune response in both vaccination trials were similar to that seen during the course of raising known anti-lambda antisera; i.e., a gradual increase especially following the second and the third immunization. An adjuvant was not included in these experiments.

Pigs had low levels of anti-PCV2 antibody responses prior to both experiments (at 49 days of age). This observation is in agreement with the finding of a study that monitored anti-PCV2 antibody levels in a commercial pig farm from birth to market age (5 – 156 days) indicating MDPCV2A declined to a very low level between 40-72 days [87]. Since, it is impracticable to find farm pigs free of MDPCV2A due to their widely distributed nature [64, 83], we minimized the effect of MDPCV2A on the efficacy of LDP-D-CAP by using pigs at 7-wks of age with minimal levels of MDPCV2A. In contrast, PCV2 vaccines developed then far have been tested in pigs at 2-4 weeks of age [60, 148, 149]. These animals assumedly have much higher levels of MDPCV2A with unknown effects on the vaccine efficacy. In this respect, we also ensured that both the control and vaccinated pigs had the similar low level of MDPCV2A at the beginning of the experiment thorough randomization based on ELISA readings on sera collected prior to immunization.

Anti-PCV2 immune responses to LDP-D-CAP were shown in both the pilot experiment with three pigs and the second trial with a larger numbers of pigs. The latter experiment, which was a better reflection of the immune response to the vaccine candidate, showed significant anti-PCV2 antibodies after the primary dose. In contrast to anti-lambda response, subsequent dosing did not improve anti-PCV2 antibody levels further, in fact, these were somewhat lowered following the third immunization in both experiments. Anti-PCV2 antibody levels in the controls that received unmodified phage were low and stable throughout the experiment. The decline in the anti-PCV2 immune response following the third vaccination leads us to question whether the high level of carrier specific immunity at this stage of the experiment contributed to a suppressor effect. In contrast, March et.al. [240] reported in their phage DNA vaccine experiment that there was no suppression in the development of vaccine antigen specific immune responses even after the fourth vaccination. The different observations can be attributed to differences in the nature of the two vaccines. We employed a display vaccine where the amount of vaccine antigen would degrade over time, while the phage DNA vaccine antigen is expressed through a mammalian expression system over a period that could enable it to overcome a suppressive effect by carrier specific immunity. None of the other phage display vaccine studies [228, 231, 239, 262] have investigated the effect of carrier specific immunity towards the development of vaccine antigen specific immune response though they employed a much higher dose (2×10^{10} - 1×10^{13} per mouse) of vaccine phage particles per Kg b.wt.

We performed western blots to confirm the findings of the ELISA, that vaccine phage particles display PCV2-Cap epitopes and that they induce anti-PCV2 antibodies in pigs. The blot stained with anti-PCV2 polyclonal antiserum from a gnotobiotic pig showed a clear band with the vaccine phage. Consistent with our results, lambda coat proteins for the unmodified lambda control did not react with this serum. The reaction with serum from a vaccinated pig showed the staining for D-CAP on the phage display vaccine (24.6 kDa-calculated for 221 aa) and PCV2-Cap (28 kDa observed [48]; 25.9 kDa–calculated for 233 aa) on PCV2 infected cell lysate indicating induction of anti-PCV2 immunity. This serum reacted with lambda coat proteins both on the vaccine and unmodified phage preparation since vaccination induced anti-lambda antibodies as well. Serum collected prior to vaccination did not react with the PCV2-CAP from the PK-15 cell lysate. However, pre-immune serum stained D-CAP and another phage band lightly (possibly a D-CAP trimer) on the phage vaccine. This reactivity with the phage D-CAP

protein could result from a low level of anti-PCV2 antibodies existing prior to vaccination. We have consistently observed D-CAP band ran at a relatively higher level than the expected MW. This finding is in consistent with a earlier observation [272] that lambda proteins migrate relatively more slowly in gels with high bisacrylamide/acrylamide ratios (8:300) which we used in our experiments.

We addressed an important question, namely, whether the LDP-D-CAP can induce neutralizing antibodies? Both LDP-D-CAP immunized pigs tested had a considerable increase in virus neutralizing titer compared to the level prior to immunization and to a control pig that received unmodified lambda particles. This confirmed that phage particles displaying D-fusion peptides comprising the four immunodominant regions of PCV2-Cap can induce PCV2 specific antibodies. The gene encoding the D-CAP fusion peptide included a string of the immunodominant regions from the PCV2-Cap, i.e., ~50% of the amino acids, including eight out of nine biologically significant sites. Therefore we probably have included the complete repertoire of neutralizing antibody generating epitopes.

We used DTH reactions to evaluate the ability of LDP-D-CAP to induce CMI because of its importance in viral infections. DTH is an inflammatory response. It is characterized by infiltration of mononuclear cells following the presentation of antigen by Langerhans cells to sensitized Th1 cells at the site of antigen deposition [273]. We evaluated the magnitude of the reaction for specific antigens and their placebo in comparison to a severe inflammation elicited by PHA-P (positive control). Though all LDP-D-CAP vaccinated (6) and unmodified lambda vaccinated control pigs (6) were subjected to skin testing, biopsies could only be collected from three vaccinated and five controls. All vaccinated pigs (3 of 3) showed responses to PCV2 antigen compared to 2 of 4 controls that received unmodified lambda in place of the phage display vaccine. One LDP-D-CAP vaccinated pig had an intensive inflammatory response comparable to that of the positive control. All LDP-D-CAP vaccinated (3 of 3) and 3 of 5 unmodified lambda vaccinated control pigs reacted to lambda phage antigens. Importantly, these data confirm the development of cell-mediated immunity to recombinant PCV2-Cap fusion protein (D-CAP) following phage display vaccination. Cellular immunity is necessary in combating PCV2 replication in affected pigs [134]. The reason that some control pigs reacted to

PCV2 antigens is probably due to the presence of sensitized Th1 cells formed prior to our vaccination trial.

Apart from the knowledge that phage particles are safe in eukaryotes [187, 210], substantial evidence was generated in the immunization trial to indicate that LDP-D-CAP was safe. First, LDP-D-CAP production procedure did not involve any inactivation procedure and the final testing preparation contained only the purified non-viable lambda antigens. In contrast, all commercial PCV2 vaccines presently available in the North America are subjected to an inactivation procedures and probably contained unpurified PCV2 antigens [96, 274]. Secondly, we did not observe any untoward injection site reactions even with a dose of 1×10^{10} phage particles per Kg b.wt. Thirdly, we have observed a steady increase in body weights of immunized pigs, ~ 17 Kg per two weeks indicating good general health without probable systemic reactions. Given the fact that production of commercial PCV2 vaccines require either propagation of slow growing PCV2 in tissue culture yielding a low viral titer (~10^5 TCID50 per ml) [61, 65, 66] or expression and purification of PCV2 recombinant capsid protein [19, 275], we believe that production of lambda vaccines is highly economical because of feasibility to produce *E. coli* lysates containing high titer lambda display particles (~10^9 per ml, Tables 4.1 & 4.2). However, a meaningful comparison of the costs for the production of commercially available PCV2 vaccines to the lambda display vaccine is not possible at this stage because of several reasons. First, we have produced the display vaccine in small quantities only for our experiments and secondly, its dosage is not yet fully optimized. Finally, the price of commercially available PCV2 vaccines is not available in the public domain.

In conclusion, we have developed a potential PCV2 vaccine candidate without incorporating any adjuvant in the final preparation, and one that can be very economical.

CHAPTER 4: PREPARATION OF LAMBDA DISPLAYING FUSION POLYPEPTIDES

1. Introduction

Lambda phage display technology is considered a better method compared to other bacteriophage display systems because of its ability to yield higher display density i.e., higher percentage of lambda display fusion partner copies were linked with display peptides [188, 197, 232-237]. It has multiple uses, notably including gene therapy [276], developing diagnostic reagents [241, 242] and vaccines [277]. There are two basic methods of displaying foreign peptides on phage particles: inserting the target gene into the phage genome, or their expression through plasmids carrying genetic codes for the fusion peptides of interest [189, 214]. The latter method is preferred as it yields mosaic phage particles i.e., particles bearing both wild type and those fused to display peptides and, therefore, causing less impact on phage morphogenesis [278]. Also, it would not raise the common concern of using genetically modified organisms in biological systems. Further, lysogen containing lambda mutant prophage with heat- labile repressor protein provides technology for more regulated expression of fusion proteins by thermal induction [279, 280]. Heat inactivation of the repressor protein derepresses the lambda promoter inducing protein synthesis. We investigated if lambda displaying fusion peptides linked to D protein i.e., D-CAP, D-FLAG and D-GFP could be produced using this method. CAP, FLAG and GFP denote four immunodominant regions of PCV2-Cap (chapter 3, section 2.1), FLAG tag peptide [281] and Green fluorescent protein, respectively.

2. Materials and methods

2.1. Demonstration of thermo regulated expression from lambda promoter

2.1.1. Thermal induction of cell killing

E. coli 594 cells, lysogen containing mutant lambda prophage with heat-labile repressor protein, were transformed with pD-FLAG (594 [pD-FLAG]) and the viability of these cells was estimated by plating dilution of overnight culture on Tryptone broth agar (TBA, 0.5% w/v NaCl, 1% w/v Bacto tryptone and 1.1% w/v Bacto agar) at 30^{o}C, 37^{o}C, 39^{o}C and 42^{o}C. Percentage of viability at each temperature was calculated in relation to viable cell counts (cfu/ml) at 30^{o}C.

2.1.2. Thermal induction of susceptibility to lambda infection

594 [pD-FLAG] cells were cross streaked on λvir and λc172 and incubated at 30^{o}C and 42^{o}C to demonstrate inactivation of repressor at 42 ^{o}C and, therefore, that cells become susceptible to λc172 infection.

2.2. Protein extraction

E. coli 594 strain with lambda heat-labile repressor was transformed with pD-CAP, pD-FLAG and pD-GFP separately. A single colony from each culture of transformed cells was grown overnight at 30^{o}C in 25 ml of Tryptone broth (0.5% w/v NaCl and 1% w/v Bacto tryptone) containing 50 µg/ml of Ampicillin. Nine flasks, each containing 25 ml of LB medium (0.5% w/v NaCl, 0.5% w/v Bacto yeast extract, 1% w/v Bacto tryptone) and the respective antibiotic were inoculated with each culture at 1:20 dilution and grown at 30^{o}C until OD_{575} reached 0.4. Four of the flasks per culture, each designated 20 min, 1 hr, 1.5 hr or 4 hr were transferred to two shaking water baths each set at either 39^{o}C or 42^{o}C to induce protein expression and incubated for designated time periods. The flasks were removed after elapsing appropriate time periods, followed by protein extraction. One inoculated flask from each culture remained at 30^{o}C for a further 1.5 hr to serve as the control for thermal induction of D-fusion protein expression. A culture of untransformed *E. coli* grown at either 39^{o}C or 42^{o}C served as the control for plasmids expressing D-fusion proteins. Cells induced for protein expression and non-induced and untransformed control cultures were harvested by centrifugation (Beckman, J2-21) at 6000 rpm for 10 min. Cell pellets were resuspended in 0.5 ml of TES buffer (0.02M Tris PH 7.6, 0.002M EDTA PH 7.6 and 0.15M NaCl), added with 0.5 ml of a lysing mixture (0.002M Tris PH 7.6, 0.04M EDTA PH 7.6, 0.15M NaCl and 0.87M SDS) and boiled for 3 min. Protein extracts were cooled to ambient temperature and added with proteinase inhibitor cocktail (Roche Diagnostics Canada, Laval, Quebec) as per the manufactures recommendation. Aliquots were stored at -70^{o}C. Protein concentration was estimated using NanoDrop® ND-1000 Spectrophotometer (NanoDrop Technologics, Inc., Wilmington, DE, USA).

2.3. Demonstration of D-fusion proteins in *E. coli* cell extracts by Western blots (WB)

Cell extracts were adjusted to contain an equal amount of total protein (delivering 40 µg per well of polyacrylamide gel) in phosphate buffered saline (PBS, 0.0036 M KCl, 0.0014 M

KH_2PO_4, 0.136 M NaCl, 0.004 M Na_2HPO_4, pH 7.4) and boiled in SDS-PAGE sample buffer (0.0625M Tris pH 6.8, 4.65% (w/v) SDS, 20% (v/v) glycerol, 10% (v/v) β-mercaptoethanol, 0.025% (w/v) bromophenol blue) for 5 min and separated by 12% polyacrylamide gel in Mini-PROTEAN 3 electrophoresis cell (Bio-Rad Laboratories, Hercules, CA, USA) along with protein markers (Bio-Rad Laboratories, Hercules, CA, USA). Proteins were transferred to nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA, USA) at 400 mA for 90 min. The membranes were blocked overnight in Tris-buffered saline (TBS, 20 mM Tris, pH 7.5, 0.5M NaCl) containing 0.1% $Tween_{20}$ and 5% skimmed milk. The blots were washed twice in TBS containing 0.04% $Tween_{20}$ (TBST) for 5 min prior to reaction with specific primary antibody for the D-fusion protein. To demonstrate D-CAP, a blot was reacted with anti-PCV2 polyclonal antiserum, from a gnotobiotic pig, at a dilution of 1:200 in TBST at ambient temperature for 1 hr. After washing the membrane 3X (for 5, 15, and 5 min) with TBST, it was incubated with biotinylated protein A (Invitrogen, Carisbad, CA, USA) at a dilution of 1:1500 in TBST for 1 hr at ambient temperature. The membrane was rinsed 3X (as above) with TBST. To demonstrate D-FLAG, the blot was reacted with anti-FLAG biotinylated BioM5 monoclonal antibody (Sigma-Aldrich Inc., St. Louis, MO, USA) at 2 µg per ml in TBST at ambient temperature for 1 hr followed by washing the membrane 3X (for 5, 15, and 5 min) with TBST. Then both D-CAP and D-FLAG membranes were incubated with streptavidin-alkaline phosphatase (Bio-Rad Laboratories, Hercules, CA, USA) at a dilution of 1:1500 in TBST for 1.5 h at ambient temperature. The blots were washed 3X in DW for 15 min and the reaction was visualized by applying freshly prepared BCIP/NBT reagent (Invitrogen, Carisbad, CA, USA) as per the manufacturer recommendation. The color development was stopped by rinsing the membranes with DW.

2.4. Preparation and purification of lambda particles displaying D-fusion proteins

Preparation of lambda particles displaying D-CAP, D-FLAG and D-GFP fusion proteins was as previously described [277]. Three 4 litre flasks containing 2 litres of LB medium (0.5% w/v NaCl, 0.5% w/v Bacto yeast extract, 1% w/v Bacto tryptone), containing 0.01M $MgCl_2$ and 0.01M Tris, pH 7.6, plus 50 µg/ml of either Ampicilin or Kenamycin, were inoculated with an overnight culture of 594 cells transformed with one of the plasmids; pD-CAP, pD-FLAG or pD-GFP, and placed in a shaking incubator cabinet at 39^{o}C. When the cells reached an absorbance of

A_{575}=0.1, each culture was infected with λ*imm*434*cI* at a multiplicity of infection of 0.1. The cultures were removed from the incubator 3 hr after infection. The lysate was clarified by centrifugation at 10,000 rpm for 10 min in JLA 16.25 rotor in Avanti J-E centrifuge (Beckman Coulter, Inc., Fullerton, CA, USA) and the supernatant solutions were held overnight at 4^{o}C. NaCl and polyethylene glycol (PEG) MW 6000-7500 were added to 0.5 M and 4% (w/v), respectively, into the supernatant solutions, shaken into solution, and held overnight at 4^{o}C. The solutions were centrifuged at 8,000 rpm for 30 min and the enriched phage pellets were gently resuspended in buffer (TM, 0.01 M Tris, 0.01 M $MgSO_4$ .7H_2O and 0.1 M NaCl, and pH 7.5). NaCl and PEG were added to 0.5 M and 4% (w/v), respectively, into pooled resuspended phage pellet, and after holding the suspension overnight at 4^{o}C, it was repelleted at 8,000 rpm for 30 min. The second phage-enriched pellet was gently resuspended in TM buffer, solid CsCl was added to a density of 1.5 g/cc, and the solution was subjected to centrifugation at 45K in an SW 60 Ti rotor in L8-M ultracentrifuge (Beckman Coulter, Inc., Fullerton, CA, USA) for 48 hr. The phage bands were recovered and rebanded.

2.5. Viable phage counts by plaque assay

Ten-fold dilutions of either phage lysate or banded phage display preparations were made in ϕ80 buffer (0.01M Tris and 0.1M NaCl, pH 7.8). One tenth of an ml (0.1 ml) of each dilution was mixed with 0.25 ml overnight broth culture of *E. coli* strain 594, R594 F *lac*-3350 *galK 2 galT22 rpsL* 179 IN (*rrnD-rrnE*) l [264] in a sterile test tube to what was added 3ml of top agar (0.5% w/v NaCl, 0.65% w/v Bacto agar, 1% w/v Bacto tryptone) maintained in liquid at 40^{o}C. The mixtures were immediately poured on Tryptone broth agar and incubated at 37^{0}C overnight. Clear plaques on plates corresponding to three consecutive dilutions were counted and average plaque count (pfu/ml) was calculated accordingly.

2.6. Total phage counts

Total phage counts, i.e., including both live and dead phage, of banded phage display preparations were calculated based on A_{260}. One microgram of DNA is calculated to be equivalent to 1.2 x 10^{10} lambda particles (unpublished data).

2.7. Demonstration of phage displaying D-CAP by indirect ELISA (iELISA)

The middle and bottom bands of lambda displaying D-CAP and an unmodified lambda phage preparations were dialysed for 3 hr each in 1000X volume of phage dialysis buffer (PDB, 0.3 M NaCl, 0.01 M Tris and 0.05 M $MgCl_2$, pH 7.8) and then ELISA coating buffer (Na carbonate 0.05M, pH 9.2). The preparations were serially diluted in coating buffer and the starting dilution was adjusted to contain 3×10^{10} phage particles per 100µL. Duplicate wells of an ELISA plate were coated with 100µL of each dilution from the respective phage preparation and incubated overnight at 4^{o}C. Coating buffer was then removed and plates were washed (5X) with PBST. Blocking buffer was added at 200 µL per well and plates were incubated for 30 min at 37^{o}C. Blocking buffer was removed and the plate was washed (5X) with PBS containing 0.05% $Tween_{20}$ (PBST). Anti-PCV2 polyclonal from a gnotobiotic pig was dispensed at a dilution of 1:100 in PBST, 100 µl per well, and incubated at 37^{o}C for 1 hr. After washing (5X) in PBST, biotinylated protein A (Invitrogen, Carisbad, CA, USA) was added at 100 µl per well at a dilution of 1:5000 in PBST, and the plate was incubated at 37^{o}C for 1 hr. The plate was washed (5X) in PBST and 100 µl per well of streptavidin-peroxidase conjugate (ABC reagent, Vector), diluted in PBST according to the manufacturer's recommendation was added and the plate was incubated at 37^{o}C for 30 min. 3.3', 5.5'-tetramethylbenzidine (TMB) substrate (KPL Inc., Gaithersburg, Maryland, USA) was added at 100 µl per well, after washing the plate (5X) with PBST. The reaction was stopped by adding 50 µl per well of 1M H_2SO_4 after 15 min of incubation at ambient temperature. The plate was read at 450 nm.

2.8. Demonstration of phage displaying D-CAP by Western blot

Two dialysed phage preparations, middle band of lambda displaying D-CAP and unmodified lambda preparations, and a PCV2 infected cell lysate as the positive control for PCV2-Cap were adjusted to contain equal amount of total protein and boiled in SDS-PAGE sample buffer for 5 min and separated by 12% polyacrylamide gel in Mini-PROTEAN 3 electrophoresis cell (Bio-Rad Laboratories, Hercules, CA, USA) along with protein markers (Bio-Rad). Proteins were transferred to nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA, USA) at 400 mA for 90 min. Blots were reacted with one of anti-PCV2 polyclonal from a gnotobiotic pig, serum from a conventional pig immunized with lambda displaying D-CAP or anti-lambda polyclonal from a conventional pig, at a dilution of 1:200 in TBST at

ambient temperature for 1 hr, to demonstrate D-CAP expression in the display preparation, estimate D-CAP:wild type D ratio, and to demonstrate lambda regular proteins respectively. After washing the membrane 3X (for 5, 15, and 5 min) with TBST, it was incubated with biotinylated protein A (Invitrogen, Carisbad, CA, USA) at a dilution of 1:1500 in TBST for 1 hr at ambient temperature. The membranes were rinsed 3X (as above) with TBST, incubated with streptavidin-alkaline phosphatase and color development steps were followed as described under 2.3.

2.9. Electron microscopy

Electron microscopy images were made from lysates and CsCl purified lambda display preparations by Dr. Hans-W. Ackermann, Department of Medical Biology, Faculty of Medicine, Laval University, Quebec, Canada.

3. Results

3.1. Demonstration of thermoregulated expression from lambda promoter

594 [pD-FLAG] cells are viable at 30^{o}C, 37^{o}C and 39^{o}C but not at 42^{o}C (Fig. 4.1a) indicating inactivation of heat-labile lambda repressor at 42^{o}C which derepresses (induces) lambda promoter, leading to cell killing. The same principle was illustrated in another experiment. 594 [pD-FLAG] cells were viable when cross streaked on λc172 at 30^{o}C (Fig. 4.1b) but became susceptible at 42^{o}C (Fig. 4.1c) due to heat inactivation of the repressor which otherwise maintained lysogeny and immunity against homologous lambda (λc172) infection. As expected, cells were killed by λvir at both temperatures (Fig. 4.1b & 4.1c).

3.2. Demonstration of D-CAP and D–FLAG fusion proteins in *E. coli* cell extracts by WB

In order to demonstrate expression of lambda D-fusion proteins in *E. coli* following thermal induction, total protein extracts collected after varying durations at respective temperatures were separated and reacted with specific antibodies. The blot reacted with anti-PCV2 polyclonal from a gnotobiotic pig (Fig. 4.2a) shows bands for D-CAP (24.6 kDa) with gradual increase in intensity beginning from the extract corresponding to 20 min through 4 hr of incubation at 42^{o}C. Similarly, Fig. 4.2B demonstrates bands for D-FLAG (20.7 kDa) with

increasing intensity beginning from 20 min to 4 hr incubation at 39^{o}C. Neither non-induced nor non-transformed cultures yielded bands in these assays.

3.3. Monitoring bacterial growth and viable phage counts during preparation of lysates of lambda displaying D-fusion proteins

Bacterial growth and viable phage counts (pfu/ml) were monitored during preparation of lysate of lambda displaying D-CAP are presented in Figure 4.3; time points "a" through "g". Actively growing cells containing pD-CAP were infected with λ*imm*434*cI* at ~0.1 OD_{575} (point b & arrow). Release of progeny viruses was evidenced as early as 1 hr post-infection (point c) while bacteria continued to multiply. Cells reached the maximum growth at 2.0 hr post-infection (point d) at which point phage titer had also increased by ~1.5 Log_{10} compared to point c, followed by a sharp drop of lysate turbidity within the next 1 hr period (up to point e, 3 hr of post-infection) due to cell lysis. This cell lysis was coincided with the burst of progeny viruses, as evidenced by the highest phage titer at point e. Lysis of cells progressed slowly for another 2 hr until it almost plateaued by 5 hr of post-infection (point e through g) during which time phage titer also dropped by ~0.5 Log_{10}.

3.4. Viable (pfu/ml) phage titers in lysates and purified lambda display preparations

Phage titers were estimated in three sets of bulk volumes of lambda D-CAP lysates at two time points: immediately after preparation, and after 5-12 days (Table 4.1). Titers dropped by a factor of 1.5 to 5.8 during this period. Table 4.2 illustrates titers at each step of lambda display preparations. Each lysate had a high initial titer (~10^9 pfu/ml) which was increased ~2-15 times following each PEG pelleting. In this phage concentration process, titers reached a maximum of 10^{11} to 10^{12} pfu/ml range prior to CsCl gradient centrifugation. Viable phage titers dropped dramatically by several Log_{10} in each of the preparation following CsCl banding.

3.5. Banding pattern of lysates containing lambda displaying D-CAP expressed in two different expression systems

Lysates containing lambda displaying D-CAP were prepared using two expression systems i.e., constitutively and by thermal induction. In both occasions, particles were separated

in to three bands on the CsCl gradient (Fig. 4.4a & 4.4b, respectively). Bands were labelled as top, middle and bottom depending on the location of the tube.

3.6. Characterization of phage bands of lambda displaying D-fusion proteins

3.6.1. Banding pattern, densities, viable and total phage titers of lambda displaying D-fusion proteins after first CsCl purification

Banding patterns of the three display preparations i.e., D-CAP, D-FLAG and D-GFP, are presented in Fig. 4.5. While lambda displaying D-CAP yielded three bands (Fig 4.5a), D-FLAG (Fig 4.5b) and D-GFP (Fig 4.5c) were separated into a single band. Visual observation, densities and viable phage counts were recorded for phage bands on each of preparation. On D-CAP, the top band was faint and contained 6.85×10^5 pfu/ml lambda particles with a density of 1.29 g/cc (Fig. 4.5a). The middle band was creamy, wide, loosely arranged, and contained 1.88×10^6 pfu/ml virus particles with a density of 1.42 g/cc. The bottom band was blue, compact, and contained 1.6×10^9 pfu/ml virus particles with a density of 1.5 g/cc. Though viable phage titers in these phage bands were significantly lower compared to titers prior to CsCl gradient (Table 4.2), the number of total phage particles recovered in each band of D-CAP preparation was high (top, middle and bottom bands contained 1.7×10^{12}, 9.33×10^{12} and 4.4×10^{12} particles per ml, respectively) and, therefore, comparable or higher titers were present prior to banding.

D-FLAG had a thin blue band containing 6.95×10^3 pfu/ml particles with a density of 1.53 g/cc (Fig. 4.5b) while D-GFP formed into a creamy and very dense band (Fig. 4.5c) containing 3.76×10^9 pfu/ml particles with a density of 1.47 g/cc.

3.6.2. Demonstration of phage displaying D-CAP by ELISA

The ELISA showed higher reactivity throughout a series of middle phage band dilutions with anti-PCV2 polyclonal from a gnotobiotic pig compared to corresponding dilutions containing equal number of lambda particles from the bottom band of D-CAP or unmodified lambda preparations (Fig. 4.6). This indicates the presence of more D-CAP protein in the middle phage band and therefore, it was used as a vaccine candidate to immunize pigs.

3.6.3. Demonstration of phage displaying D-CAP by WB

The blots reacted with either anti-PCV2 polyclonal from a gnotobiotic pig (Fig. 4.7a) or serum from a conventional pig immunized with lambda displaying D-CAP (Fig. 4.7b) revealed the D-CAP (arrow) on the lambda display particles (LDP) (lane 3) and the PCV2-Cap (triangle) on the PCV2-infected cell lysate (lane 2), respectively. The arrow with double heads on lane 3 of Fig. 4.7b showed wild type D in the lambda D-CAP display preparation. There was no corresponding D protein band on the unmodified lambda proteins (Fig. 4.7b, lane 4). The blot containing proteins from an unmodified lambda preparation reacted with anti-lambda polyclonal from a conventional pig (Fig. 4.7c). They include a tail and a head protein (unnamed) of 74 and 56 kDa respectively, the major head protein E (37 kDa), the major tail protein V (32 kDa) and the head protein D (11 kDa).

In order to estimate display density of D-CAP in the display vaccine preparation, the intensity of D-CAP (arrow) and D-wild type (arrow with double heads) bands on the lambda D-CAP display preparation (Fig. 4.7b, lane 3) was measured using a commercial software package (Northern ECLIPSE, 6.0; Imaging Software, Empix Imaging Inc., Mississauga, Ontario, Canada) and revealed D-CAP (61.5%):D-wild type (38.5%). An area on the D-wild type band was first demarcated at the same level corresponding D band shown in the Fig 4.7c prior to measure intensity of the band.

3.6.4. Electron microscopy of lambda displaying D-CAP

Electron microscopy on both crude lysate (Fig. 4.8a) and CsCl purified lambda displaying D-CAP i.e., middle phage band (Fig. 4.8b) illustrates tailless heads compared to intact particles in an unmodified lambda lysate (Fig. 4.8c). Arrows and triangles in lambda D-CAP preparations (Fig 4.8a, b) indicate heads containing DNA, and proheads i.e., prior to DNA encapsidation, respectively. Heads were hexagonal and bigger (~60 nm in diameter) compared to round and smaller proheads (~50 nm in diameter). Purified lambda D-CAP preparation (Fig. 4.8b) shows well separated tailless heads with improved clarity compared to crude lysates (Fig. 4.8a, c).

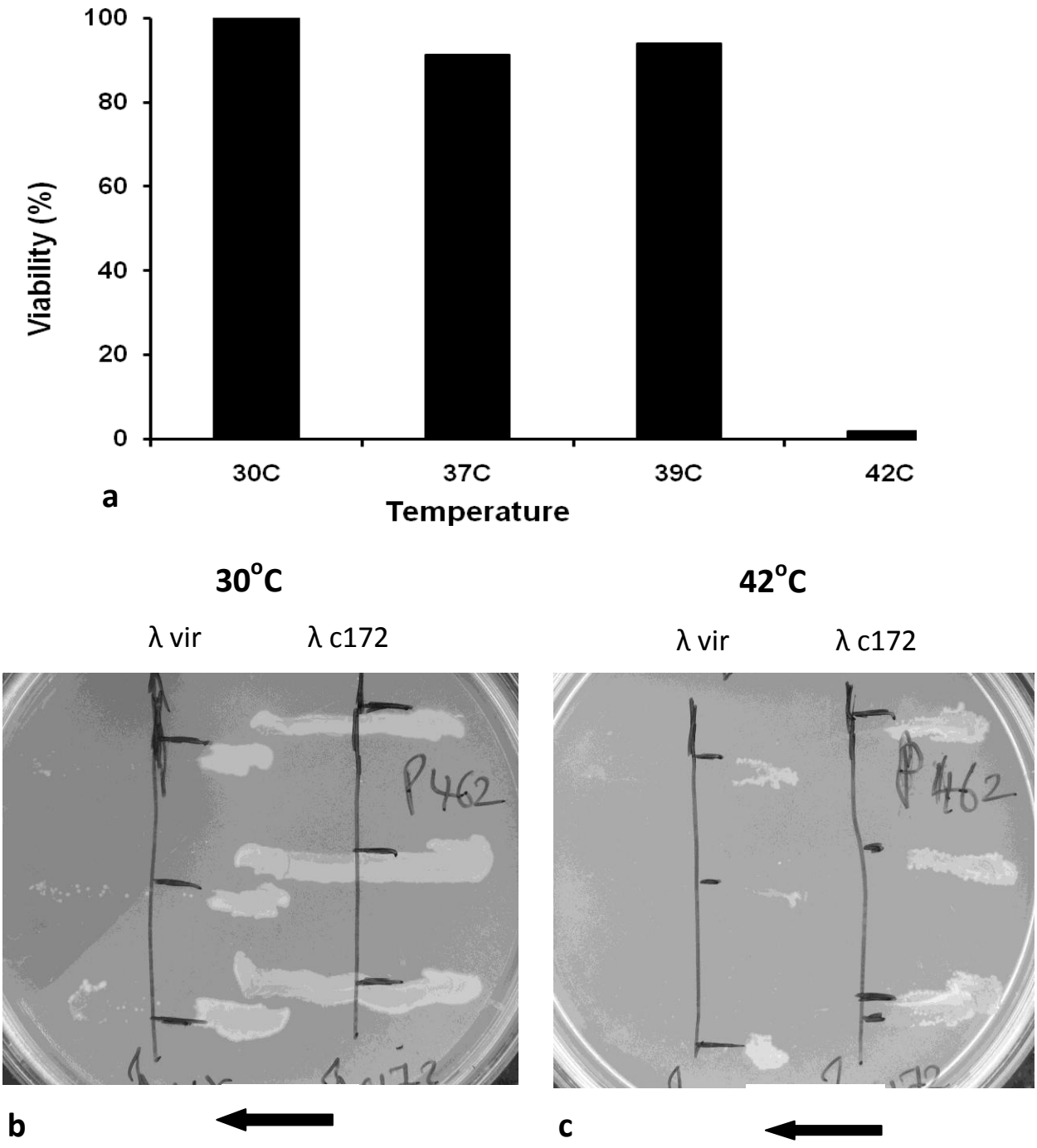

Fig.4.1 Thermal induction inactivates heat-labile lambda repressor as manifested by cell killing or susceptibility to a homologous lambda infection: (a) lysogen used to transform plasmids coding for D-FLAG were killed at 42°C, (b) immune to homologous lambda infection (λc172) at 30°C but (c) become susceptible for the infection at 42°C. Arrows indicate direction of cross streaking.

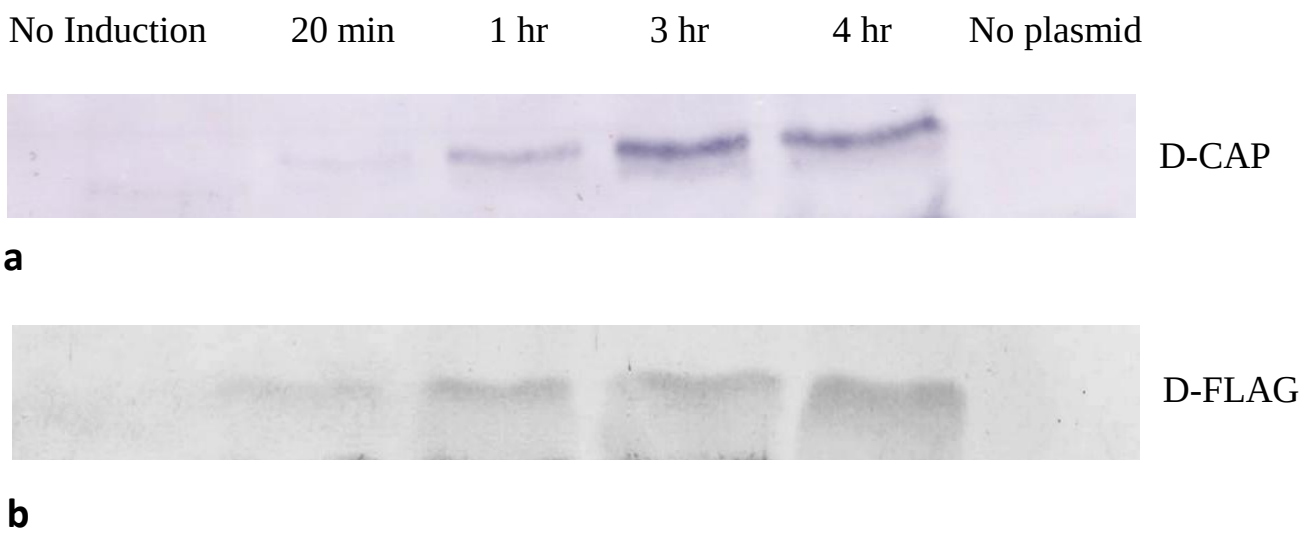

Fig. 4.2 Western blots demonstrating expression of D-CAP and D-FLAG in *E. coli* transformed with respective plasmids by thermal induction for varying periods of incubation: (a) D-CAP at 42^{o}C and (b) D-FLAG 39^{o}C. "No induction" corresponds to extracts from cells containing the plasmid but not thermally induced. "No plasmids" indicate that the cells were not transformed with plasmids but were thermally induced. Other lanes are labelled by duration of incubation.

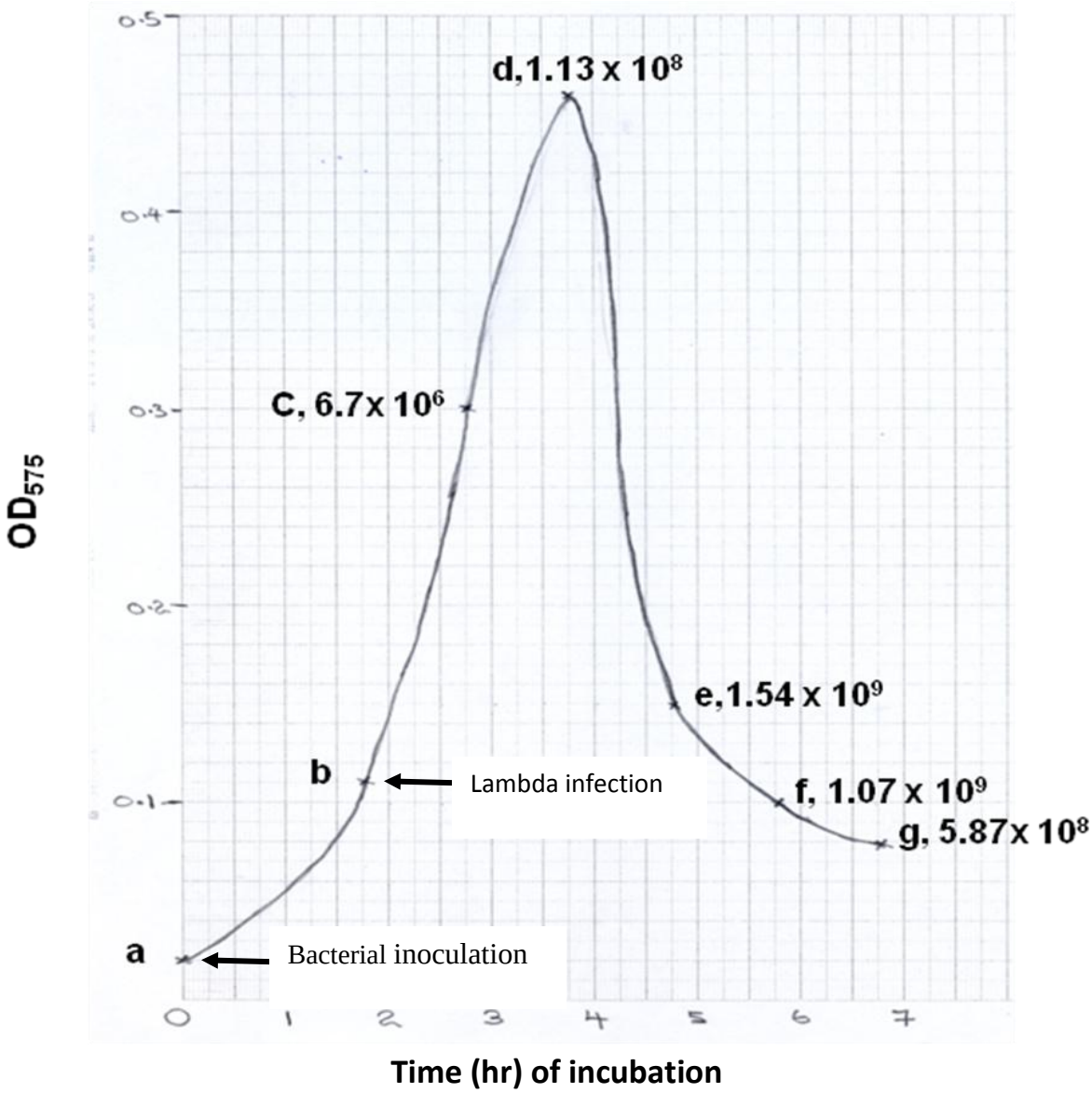

Fig. 4.3 Growth curve of *E. coli* transformed with pD-CAP during the preparation of lysate containing lambda displayed D-CAP at 39°C. Growth medium was inoculated with *E. coli* at point a. Optical densities (OD_{575}) and viable phage titers (pfu/ml) were measured from point a through g. Time of phage infection (arrow) and titers thereafter are indicated along with the corresponding stage of incubation.

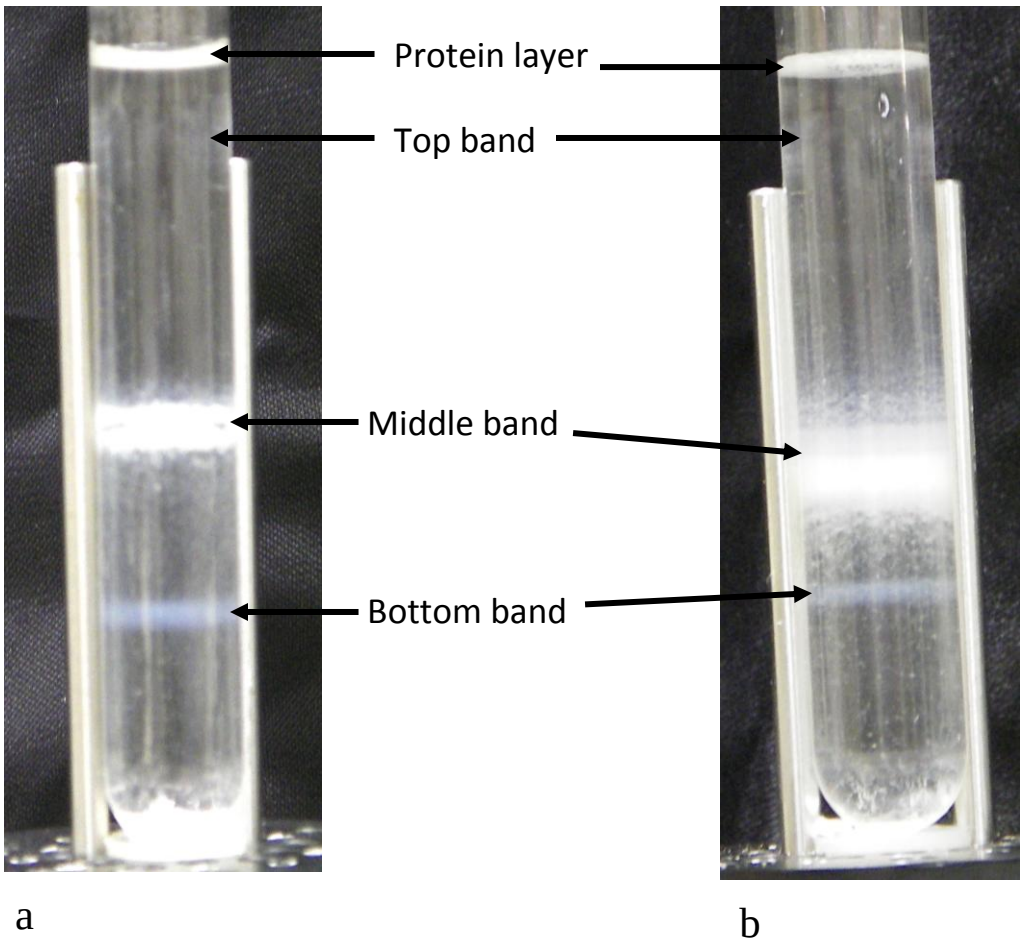

Fig. 4.4 The banding pattern of lambda displayed D-CAP in CsCl gradients produced using two expression systems: (a) λimm434*cI* infection in *E. coli* lacking thermo regulatory promoter activity transformed with pD-CAP (D-CAP expressed constitutively) (b) λimm434*cI* infection in *E. coli* containing thermo regulatory promoter transformed with pD-CAP (D-CAP expressed by thermal induction). Phage bands were identified by their location in the tube. The top white layer was protein excluded from the gradient.

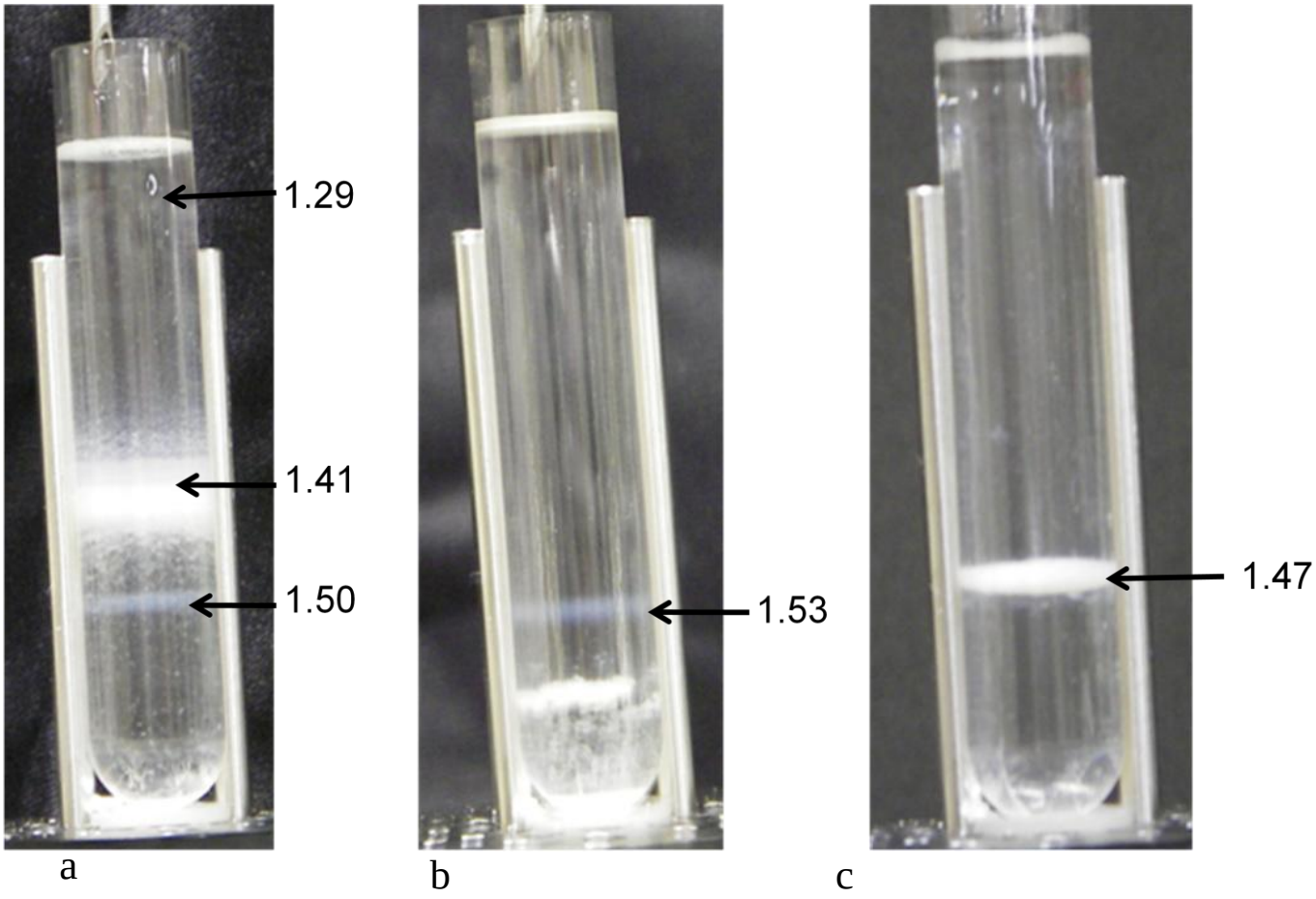

Fig. 4.5 The banding pattern of lambda displaying (a) D-CAP (b) D-FLAG and (c) D-GFP in CsCl gradients produced by lambda infection in thermally induced *E. coli* containing respective plasmids at 39^{o}C. The densities of each band are indicated by arrows.

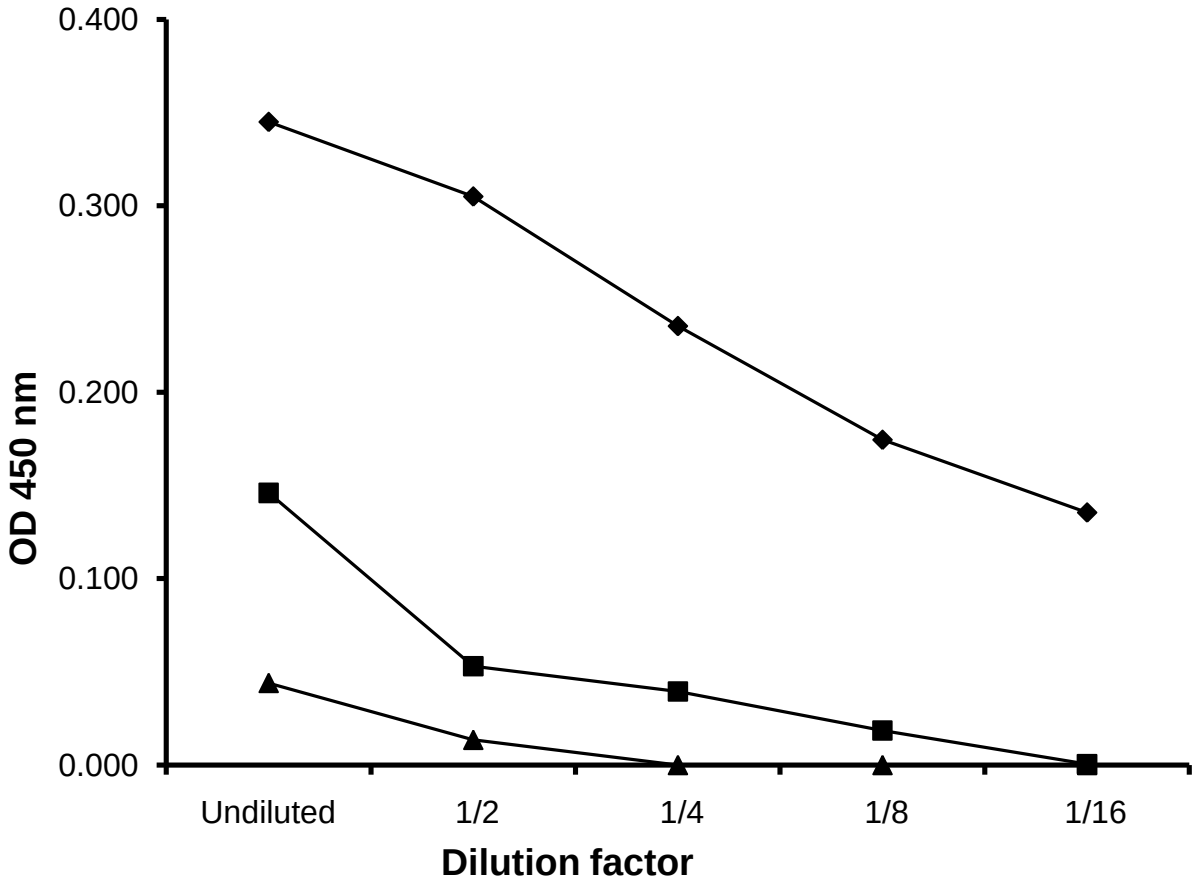

Fig. 4.6 ELISA measuring phage displayed D-CAP fusion protein using anti-PCV2 polyclonal antiserum from a gnotobiotic pig. Serial dilutions of middle (◆) and bottom bands (■) of lambda D-CAP preparation along with unmodified lambda (▲) contain equivalent number of phage particles. The number of phage particles coated in undiluted preparation was 3×10^{10} per well.

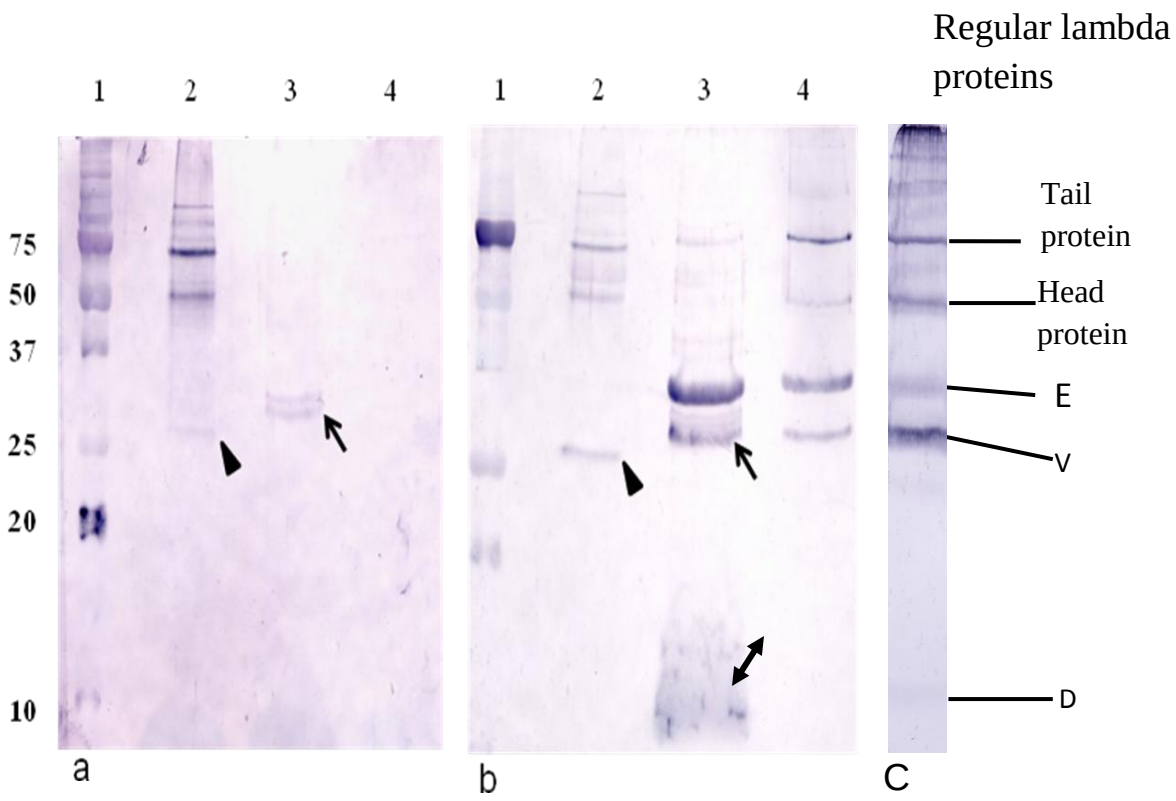

Fig. 4.7 Western blots demonstrating lambda displayed D-CAP and other major lambda proteins: (a) blot reacted with anti-PCV2 polyclonal antiserum from a gnotobiotic pig, (b) anti-D-CAP polyclonal antiserum from a conventional pig and (c) anti-lambda polyclonal antiserum from a conventional pig. Lanes; 1) Protein mass marker, 2) partially purified PCV2 antigen from PK15 infected cells, 3) LDP-D-CAP from heat-disrupted phage particles, and 4) similarly disrupted unmodified phage particles. Arrow, triangle and arrow with double heads indicates D-CAP, PCV2-Cap (positive control) and lambda D proteins respectively. Major lambda protein bands are identified on the blot c.

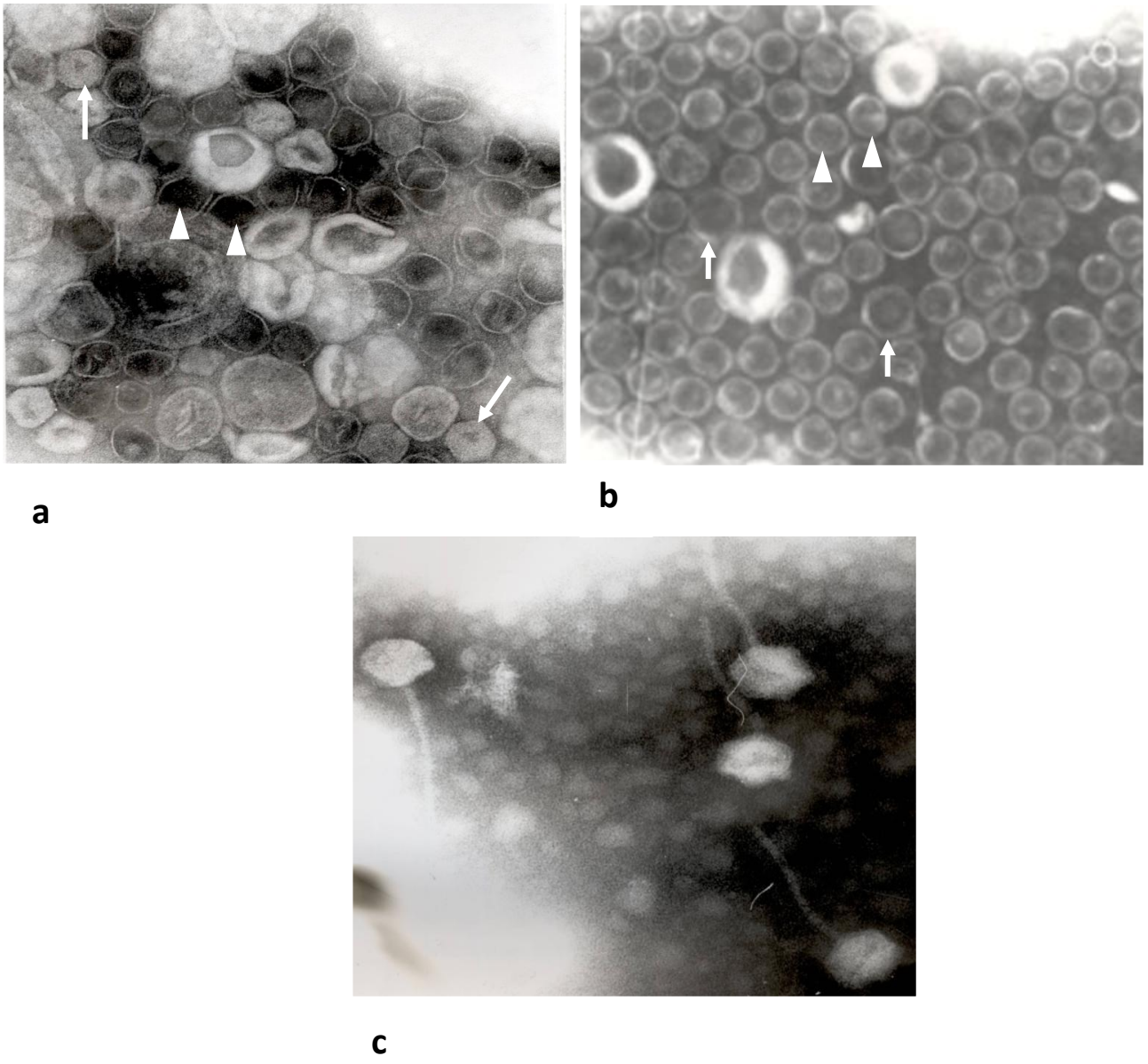

Fig. 4.8 Electron microscopy graphs showing lambda displaying D-CAP: (a) crude lysate and (b) twice CsCl purified middle band of lambda D-CAP preparation demonstrating tailless heads in comparison to (c) intact particles in an unmodified lambda lysate. Arrows and triangles indicate hexagonal relatively bigger heads and rounded smaller proheads, respectively. Magnification ×148,500.

Table 4.1

Monitoring of phage titers (pfu/ml) in lysates of lambda displayed D-CAP stored at 4^{o}C.

Lysate ID & volume	Original titer on the day of preparation	Subsequent titration	Time gap (day) between titrations
Batch A (1L)	9.6×10^{8}	6.0×10^{8}	12
Batch B (2L)	4.6×10^{9}	2.6×10^{9}	7
Batch C (4L)	2.6×10^{9}	4.5×10^{8}	5

Table 4.2

Titers (pfu/ml) of phage displaying fusion proteins after each step of the preparation.

Phage display preparation	Lysate	Post 1st PEG Pelleting	Post 2nd PEG Pelleting	Post 1st CsCl purification	Post 2nd CsCl purification
D-CAP	9.54×10^{9}	2.8×10^{11}	5.52×10^{12}	[a]1.88×10^{6}	[a]3.75×10^{5}
D-FLAG	6.75×10^{9}	1.95×10^{11}	1.8×10^{12}	6.95×10^{3}	[b]N
D-GFP	5.00×10^{9}	7.2×10^{10}	9×10^{11}	3.76×10^{9}	[b]N
Unmodified	8.62×10^{9}	2.97×10^{10}	2.91×10^{11}	1.4×10^{9}	[b]N

[a] Middle phage band

[b] Not titrated

4. Discussion

This study was undertaken to investigate the feasibility of producing a lambda display preparation using a safe method with, an intention of application in biological systems. We chose to express display peptides through plasmids, rather than cloning extraneous genes into lambda that could propagate in the event of escaping into the environment. In our method, particles displaying respective fusion peptides were produced only when the lysogen containing the plasmid was infected with a specific lambda phage at a desired temperature. Further, expression of proteins through a plasmid does not necessitate each copy of lambda D protein to be fused with the display peptide, rather it yields mosaic phage particles bearing both wild type D and those fused with display peptides, causing less impact on morphogenesis and integrity of the particle [189, 278]. In our expression system, D-fusion proteins were transcribed from the plasmid. Whereas, wild type D protein was supplied via the expression of an infecting wild type lambda genome.

The nucleotide sequences for D-fusion peptides were codon optimized for *E. coli* avoiding restriction enzyme sites, sequenced, synthetically made and included in a set of commercial plasmids containing antibiotic resistant cassettes and additional cloning sites. Fusion peptide sequences were codon optimized to ensure their synthesis in *E. coli* and to minimize the possibility of digestion by host enzymes [278, 282]. The display proteins (D-CAP, D-FLAG and D-GFP) were fused to the C-terminal of lambda D protein by a five-amino acid flexible linker that may allow fusion peptide to position outward and make them readily available for intended function. A similar study has used a linker with alternating proline and threonine repeats allowing independent folding of both D and the D-fusion proteins [233]. C-terminal of lambda D is preferred for fusing peptides because of higher display density compared to N-terminal fusions [238].

Our initial experiments confirmed thermal induction of lambda promoter. This system has the ability to control expression of fusion peptides and, thereby, avoiding spontaneous build up of potential toxic peptides in *E. coli* prior to thermal induction [188]. Heat inducible lambda functions i.e., cell killing and protein synthesis, are associated with inactivation of a heat-labile repressor protein, a product of lambda *c1*gene [279]. This protein is also responsible for maintaining the prophage (lysogeny) and, therefore, its inactivation may switch the lysogenic

state into the lytic pathway and produce progeny viruses. However, there are certain lambda mutants which do not produce progeny viruses although they were thermally induced for protein synthesis. Therefore, cell killing is suggested to be mediated by an enzyme [279]. Thermal inductions were attempted between 38-43^{o}C and non-lysogen or lysogen containing wild-type lambda do not die at these temperatures [279]. As long as prophage is maintained by this heat-labile repressor at 30^{o}C, it does not allow the bacterium (lysogen) to be reinfected with homologous lambda, i.e., λc172 (immunity), but it become susceptible at 42^{o}C with the inactivation of the repressor. The immunity is lambda type specific and, therefore, other lambda types i.e., λvir can infect the lysogen harbouring λ*c1* prophage either at 30^{o}C or 42^{o}C [279, 283].

We demonstrated expression of D-CAP and D-FLAG by WB and confirmed thermal induction of these proteins in *E. coli*. Expression of both of these display peptides was evident by specific bands of predicted molecular weight (24.6 and 20.7 kDa for D-CAP and D-FLAG, respectively) and duration of incubation dependent increase in the band size. Importantly, both the non-induced and the non-transformed cell extracts were negative for the presence of fusion polypeptides. We chose FLAG as a fusion protein because its expression was successfully demonstrated in *E. coli* as a tag protein [281]. Expression of D-CAP was attempted with the intention of displaying it fused to lambda head D protein for use as a vaccine candidate for porcine *Circovirus* 2 (PCV2). To achieve this goal, we prepared bulk volumes of lysates of lambda displaying these fusion peptides. The production procedure was standardized and demonstrated a characteristic bacterial growth curve during the process. Complete cell lysis was observed ~5 h post-infection at 39^{o}C with a burst of phage release as indicated by a sharp increase in viable titers (pfu/ml). A decline in titer on further incubation was likely be due to attachment of lambda particles to *E. coli* cell debris. Therefore, lysates were immediately clarified from cell debris and particles were subsequently concentrated by double PEG pelleting and purified by CsCl density gradient ultracentrifugation [191, 278, 284].

We were successful in obtaining phage bands in every lambda display preparation although the banding patterns were different among them. Isolated bands had a minimum viable titer of ~7 x 10^{3} pfu/ml (D-FLAG band) and total phage titer as high as ~1 x 10^{13} (middle band of D-CAP preparation). In contrast, lysates of lambda display preparation that supposedly contained particles bearing only the D-fusion peptides without wild type D did not yield any

bands because of incomplete phage morphogenesis, indicating the significance of leaving a certain number of wild type D on the phage particle [278]. In order to characterize the D-CAP bands, the two major bands i.e., middle and bottom, along with an unmodified lambda preparation were reacted with anti-PCV2 polyclonal antiserum from a gnotobiotic pig in an ELISA. Higher reactivity in the middle phage band is likely due to its higher proportion of particles displaying D-CAP fusion proteins compared to the bottom band that contained few or no particles displaying D-CAP. This could result in a higher protein:DNA ratio per particle in the middle band and, therefore, lighter (banded higher in the tube) than the bottom band with a density comparable to regular lambda (1.5 g/cc) [285]. Accordingly, the top band though very faint, may have had the highest D-CAP content. The minute quantity of this band recovered was not adequate to test this possibility. Electron microscopy provided crucial evidence for further explanation of the lambda D-CAP banding pattern. Crude lysate of this preparation contained mostly tailless heads and few intact particles, while the CsCl purified middle band contained only the tailless heads. Most heads in the middle band were either empty or only partially filled with DNA compared to unmodified lambda preparation that contained intact particles with electron dense heads, i.e., heads full with DNA. These EM data on lambda D-CAP preparation provided vital evidence of both the influence of lambda heads displaying D-CAP on their assembly with tails, and the banding pattern of this display preparation. D-CAP fused empty or partially full heads were likely higher in protein:DNA ratio per particle and, therefore, banded at relatively higher position (lower density) compared to intact and/or less D-CAP rich particles in the bottom phage band. This possibility of relatively denser intact particles separated into the bottom band was evident by its three Log_{10} higher viable phage titer compared to that of the middle band. We analysed only the CsCl purified middle band of D-CAP preparation partly because of its higher reactivity with anti-PCV2 antibodies and therefore, our interest in its use as a vaccine candidate. Further, there were technical difficulties with handling phage preparations in CsCl for EM analysis. We attempted EM on crude lysates of other display preparations but were unable to demonstrate any phage particles. It is also important to indicate that we observed the same banding pattern of lambda D-CAP preparation consistently over two batches. The first batch was prepared using cells devoid of heat-labile repressor (constitutive expression) and the other was prepared using cells with heat-labile repressor (thermally induced expression). Therefore, separation into three bands seemed characteristic of the lambda D-CAP preparation.

On further investigation of the functional significance of lambda displaying fusion peptides, one of our display preparations i.e., middle band of D-CAP which showed higher reactivity with anti-PCV2 antibodies was used in an immunization trial in pigs [277]. The pigs developed PCV2 specific humoral and cell-mediated immunity indicating functional integrity of the lambda displaying D-CAP. There are several similar vaccine candidates that have been developed though using other bacteriophage display systems [228-231].

We confirmed that our vaccine candidate contained lambda displaying D-CAP by WB using anti-PCV2 polyclonal antiserum from a gnotobiotic pig. A single band of D-CAP (24.6 kDa, calculated) was observed on the vaccine particles (purified middle band of D-CAP preparation), without staining any other lambda bands with this sera, indicated the specificity of the reaction. A similar blot reacted with sera after immunization revealed the D-CAP band along with major head protein E and wild type D on the same preparation confirming particles carried both wild type and D-fusion peptides. Interestingly, sera collected after immunization did react with all but D protein on the unmodified lambda phage preparation, suggesting a possibility of masking wild type D by D-CAP in the vaccine preparation and, therefore, not induced anti-D antibodies. In order to confirm this possibility, we compared regular/unmodified lambda proteins stained with anti-lambda polyclonal raised in a conventional pig. This experiment demonstrated bands corresponding to all lambda major proteins including D (11 kDa) providing further evidence to our explanation. Display density of D-CAP in our vaccine preparation was reasonably high (61.5%) and comparable with similar studies [238, 278]. Density of display peptides depends on their size, i.e., the smaller the size of the peptides the higher the display density [189, 286]. Electron microscopy on lambda D-CAP preparation revealed only tailless particles. This EM evidence and positive identification by anti-PCV2 polyclonal from a gnotobiotic pig confirm the band on the vaccine preparation to be D-CAP although it migrated to a position that corresponds to regular lambda V protein. On the other hand, sera from a pig vaccinated with CsCl purified lambda display particles showed anti-V reactivity. Taking all these experimental evidences into consideration, it is likely that some tail protein could also be fused with lambda heads displaying D-CAP.

Electron microscopy graphs demonstrating tailless heads in the lambda D-CAP preparation suggested of interference on lambda head assembly with their tails, most likely due

to alteration of head architecture by display peptides. Comparing EM analysis on crude lysate and purified middle band of this preparation revealed the former contained heads relatively full of DNA compared to the latter obtained by double CsCl centrifugation. This observation raises a possibility of unstable or leaky lambda heads due to their fusion with display peptides and therefore lost DNA during ultracentrifugation. We have observed loss of viable titers in the lysates of display preparations, and most dramatically after CsCl gradient centrifugation, providing further evidence to support this possibility. Intact lambda particles were reported to maintain titers much longer period (~30 years) [287] suggesting instability of particles displaying peptides, leading to loss of their infectivity. Among the three display preparation, the most dramatic loss of titer was observed in lambda D-FLAG preparation after the first CsCl gradient centrifugation.

In summary, we have attempted displaying three types of display peptides fused to lambda head D protein using a safer expression system to be applied on biological systems. We have shown display of D-CAP and the functional significance of this peptide as a vaccine candidate. We are not certain why other display preparations did not demonstrate particles by EM.

GENERAL CONCLUSIONS

1. Piglet vaccination may be negatively affected by passively acquired maternal PCV2 antibodies as suggested by my studies as well as other reports. I investigated if the parenteral administration of a prototype, adjuvanted, PCV2 vaccine to piglets at an early age could override passive immunity, and could induce acquired immunity in young piglets. My results were not conclusive. They suggested that further investigations were required that involved varying vaccine antigen doses in combination with different adjuvants to piglets with defined concentrations of maternally-derived PCV2 antibodies.

2. Preexisting anti-lambda antibodies in pigs could interfere with immune induction by a lambda phage display PCV2 vaccine candidate. Sera from 55 farm pigs were tested for anti-lambda antibodies by ELISA in comparison to known negative and positive sera. The pigs contained very low (or no) anti-lambda antibodies. This suggested that a lambda phage vaccine can be administered into pigs without the risk of suppressing the target vaccine antigen specific immune response.

3. We chose to induce lambda D-fusion peptides from plasmids within transformed *E. coli* cells. Protein extracts of the thermally induced cells were tested for expression of D-fusion peptides by Western blots. These experiments demonstrated the expression of D-CAP and D-FLAG fusion proteins from a thermally inducible protein expression system in *E. coli*.

4. Lambda particles displaying D-fusion peptides, D-CAP, D-FLAG and D-GFP, were produced by infecting thermally induced *E. coli* expressing respective D-fusion peptides with lambda and purifying the display particles by double CsCl gradient centrifugation. The phage bands were characterized based on their densities, viable titers, electron microscopy and immunological assays (ELISA and Western blots).

5. Conventional pigs were immunized with lambda particles displaying D-CAP without an adjuvant, in two separate vaccination trials. These experiments showed induction of anti-PCV2 humoral and cell-mediated immunity, and the presence of virus neutralizing antibodies. This preparation served as the first display vaccine candidate produced using phage lambda.

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